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EXPERIMENTAL CELL RESEARCH



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STUDIES ON THE SURFACE LAYERS OF OOCYTES AND MATURE EGGS OF *PSAMMECHINUS MILIARIS*

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THE presence of lipids, as well as proteins, in the surface layers of the sea urchin eggs has been demonstrated in different ways; for example, the observations with dark field illumination by Runnström (22, 23); the microscurgical experiments by Chambers (2) and Kopac (9); the studies in polarized light by Runnström and co-workers (cf. 29) and by Monroy (17); the results obtained with treatment with enzymes (20, 27, 28). However, little is known about the arrangement of proteins and lipids in the fine structure of the surface layers, and also about the modifications this structure undergoes during the maturation of the oocyte. Another question still open concerns the separate existence of one or more membranes or layers, morphologically as well as physiologically distinct, on the cell surface. It is generally admitted that at least a "vitelline membrane"¹ is present (cf. 26), but recently this has been doubted (8, 16).

The use of enzymes can contribute to a solution of such problems. In the present paper are reported some results obtained on oocytes and eggs of *Psammechinus miliaris* by treatment with proteolytic enzymes and with bee venom, whose activity on the oocytes and the eggs, as shown below, is due to its content of phospholipase A.

MATERIALS AND METHODS

The experiments were carried out on oocytes and unfertilized mature eggs of both the S- and Z-forms of *Psammechinus miliaris* (P. L. S. Müller) (11) at the Kristineberg Zoological Station, during the summers of 1952 and 1953.

Crystalline trypsin (General Biochemical Co.) containing about 50 per cent salts, and bee venom ("Bienengift" Hoffman-La Roche & Co.) have been used; in some ex-

¹ For such surface layers we shall use the nomenclature proposed by Runnström and Monné (27).

periments also chymotrypsin and activated papain, as indicated in a preceding paper (14), where the standard values of the activity of the proteolytic enzymes are given. Stock solutions containing 0.1 per cent of the substances were prepared daily in sea water and diluted as desired by addition of sea water (pH about 7.8, not corrected).

The bee venom solutions did not lose any of their activity when brought to the boiling point for a few minutes. A purified preparation, supposed to contain only phospholipase A, was obtained by the method of Gronchi (7). No qualitative difference in activity on *Psammochinus* material has been observed between such preparation and the untreated bee venom; even the quantitative activity is the same, if related to the amount of bee venom used. An attempt has been made to isolate a phospholipase A from Pankreatin Merck, according to Gronchi (6). A very low yield of active substance has been obtained, but with the same qualitative action as the bee venom. For practical reasons we mostly used the bee venom as such, and the concentrations reported in the paper refer to this substance.

The experiments were carried out at room temperature (about 22°C). The jelly layer was not removed before the treatment.

OBSERVATIONS

Treatment with proteolytic enzymes

The extreme sensitivity of the surface layers of the mature eggs to the proteolytic enzymes (trypsin, chymotrypsin and papain) has already been described, and quantitative data have been given (cf. 14).

The surface layers of the oocytes are also sensitive to proteolytic enzymes, although to a lesser extent. When the oocytes are treated with a proteolytic enzyme and then transferred to a hypotonic solution (25 parts of sea water and 75 parts of distilled water), at the beginning they always show a "membrane", similar to that of the controls, which separates from the granular endoplasm and elevates first at a few points, and then on the whole surface. This membrane is present even after extreme conditions of treatment (e.g. 24 hours in 0.1 per cent trypsin). However, if the treatment has been sufficient, a few minutes after immersion in the hypotonic solution this membrane ruptures at one point (probably at the animal pole), contracts quickly and immediately breaks down into granules, dispersed by Brownian movement. The time necessary for such breakdown is clearly longer than that reported for the mature eggs in similar conditions (14): using a 0.001 per cent solution of trypsin, the time of treatment necessary for the oocytes is 4 minutes, and only 1 minute for the mature eggs.

The modifications caused by the proteolytic enzymes on the surface layers are also indicated by the different rate of swelling in a hypotonic medium (35 per cent sea water). Fig. 1 shows such swelling for normal oocytes and

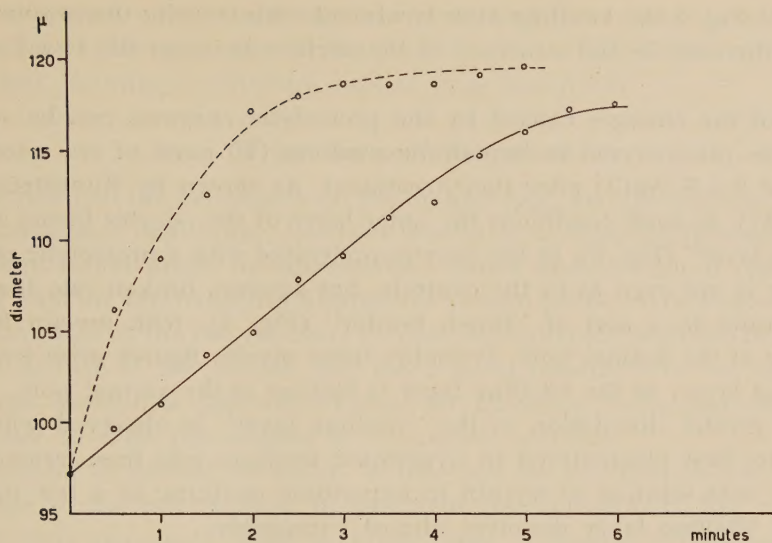


Fig. 1. Swelling of mature eggs (continuous line) and oocytes (dotted line) in 35 per cent sea water.

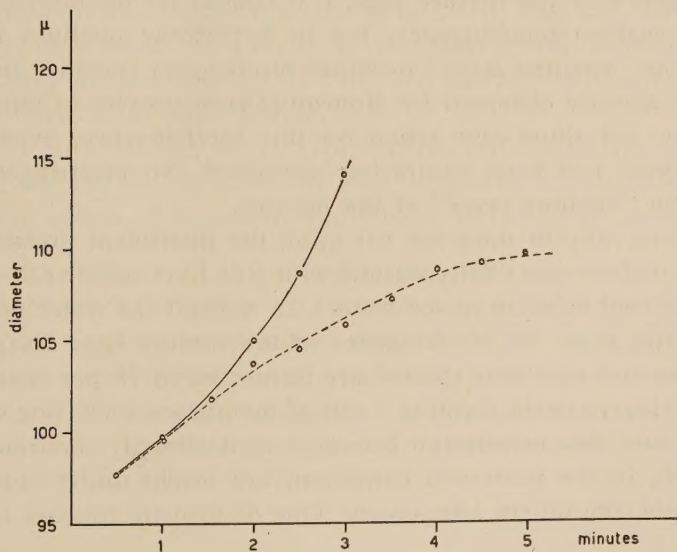


Fig. 2. Swelling of mature eggs (continuous line) and oocytes (dotted line) treated with 0.01 per cent trypsin for 4 hours and transferred in 35 per cent sea water.

eggs¹, and Fig. 2 the swelling after treatment with trypsin: the graphs point also to difference in the structure of the surface between the two kinds of cells.

Some of the changes caused by the proteolytic enzymes can be seen in the oocytes plasmolysed in hypertonic medium (20 parts of sea water and 6 parts of 2.5 N NaCl) after their treatment. As shown by Runnström and Monné (27), in such conditions the outer layer of the oocytes forms a thick "vitelline layer" (Fig. 3). In the oocytes pretreated with a proteolytic enzyme this layer is not even as in the controls, but appears broken into filaments and granules in a sort of "brush border" (Fig. 4), with myelin figures, especially at the animal pole. Probably these myelin figures arise from the underlying layer, as the vitelline layer is lacking at the animal pole.

A still greater dissolution of the "vitelline layer" is observed when the oocytes are first plasmolysed in hypertonic medium and then treated with a 0.1 per cent solution of trypsin in hypertonic medium: in a few minutes the thick vitelline layer dissolves almost completely.

Trypsin has no influence on the birefringence of the surface layers. For mature eggs it has been pointed out by Runnström, Monné and Broman (28) that the surface birefringence is not altered by trypsin: also in our experiments a treatment with 0.1 per cent trypsin for several hours does not impair the birefringence of the mature eggs. It is similar for the oocytes: normally there is no surface birefringence, but in hypertonic medium the surface underlying the "vitelline layer" becomes birefringent (positive in the radial direction, as already observed by Monroy (18) in oocytes of other species). Trypsin does not show any action on this birefringence, even when the "vitelline layer" has been completely dissolved. No birefringence is ever shown by the "vitelline layer" of the oocytes.

Furthermore, trypsin does not act upon the dichroism presented by the oocytes and mature eggs vitally stained with Nile blue sulfate (30–60 minutes in a 0.01 per cent solution in sea water). In normal sea water no dichroism is evident, and even the birefringence of the mature eggs disappears; but when oocytes and eggs thus stained are immersed in 75 per cent sea water, their surface layers swell, forming a sort of membrane enclosing an irregular endoplasm, and this membrane becomes soon strongly dichroic: the quadrants which, in the unstained condition, are bright under compensation, appear purple, the others blue-green. This dichroism appears in the same

¹ The curve for the swelling of the mature eggs is not in agreement with that of Lucké (12): probably the discrepancy is to be attributed to the different experimental conditions (*Arbacia* eggs in 70 per cent sea water in Lucké's experiments).

way even when oocytes and eggs are pretreated for 4 hours in a 0.001 per cent solution of trypsin: also in this case the cell surface swells in 75 per cent sea water, forming a structure similar to a membrane.

Treatment with bee venom

The action of the bee venom on the mature eggs, and the induced elevation of a fertilization membrane, has been described in detail by Runnström (24). The observation made by Runnström, Monné and Wicklund (29) is confirmed: that the birefringence disappears as soon as the surface layers appear to be modified by the bee venom (i.e. when a roughening begins to be evident).

The surface of the oocytes also has a great sensitivity to bee venom. Its apparent reaction is different whether the treatment takes place in normal sea water or after plasmolysis in hypertonic medium.

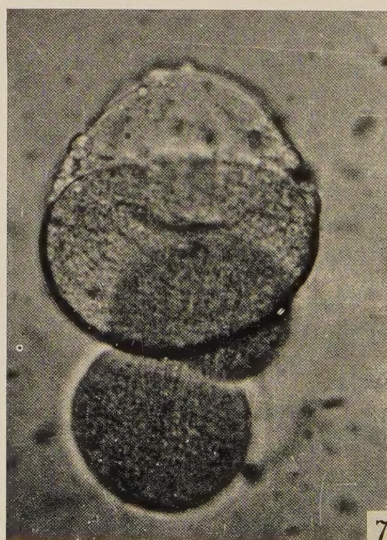
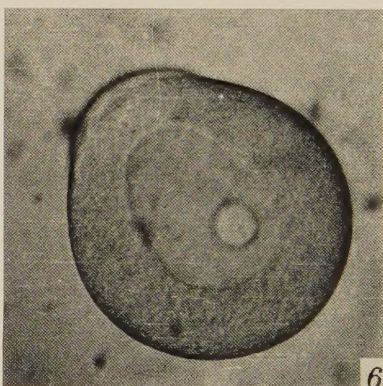
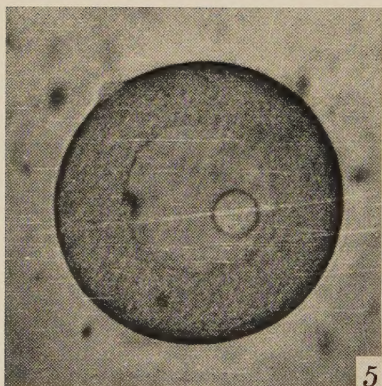
Treatment in normal sea water. In this case the oocytes cytolysed, owing to breakage of the cell surface. In sufficient concentration of bee venom (at least 0.0001 per cent), after a certain time, a hyaline blister (sometimes more than one) forms, probably at the animal pole (Fig. 5). It swells, and the granular endoplasm flows into it, together with the germinal vesicle (Fig. 6), whose nucleolus disappears. Then the surface of this exovate breaks down into granules, and cytolysis ensues: in many cases the vegetal part can round off and escape cytolysis (Fig. 7). In a weaker solution of bee venom many little blisters can form, as described by Runnström and Monné (27): often they are severed off, and the cell surface reconstitutes: cytolysis takes place later, from other blisters.

In Table I and in Fig. 8 are reported the average times necessary for cytolysis of all oocytes in different concentrations of bee venom. Table I also gives the action of the same solutions in inducing a fertilization mem-

TABLE I

Bee venom per cent	Time for cytolysis of oocytes	Induced fert. membrane in mature eggs
0.005	less than 1 minute	normal fertiliz. membr. (cytolysis)
0.003	3 minutes	incomplete fertiliz. membr. (cytolysis)
0.001	5 "	" " " "
0.00025	15 "	" " " (a)
0.0001	2 hours	no fertiliz. membr. (a)
0.00001	50 per cent cytolysis in 4 hours	" " " (a)

(a) These eggs do not cytolysed and form a normal fertilization membrane when inseminated.



brane in the eggs. It is possible to see that the oocytes are more sensitive than mature eggs to the bee venom.

In some experiments the oocytes were treated with bee venom for a short period (e.g. 7 minutes in a 0.00025 per cent solution) and washed before

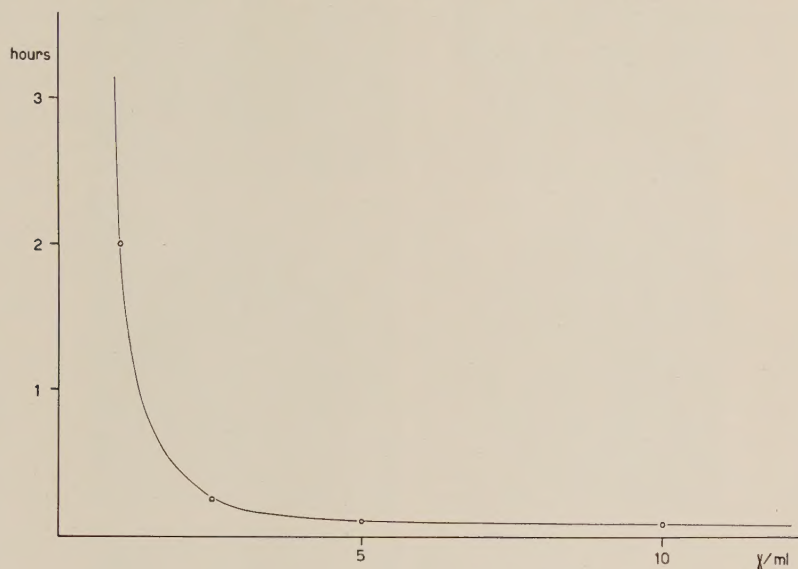


Fig. 8. Time for the cytolysis of the oocytes in bee venom.

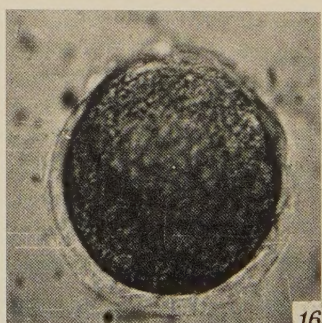
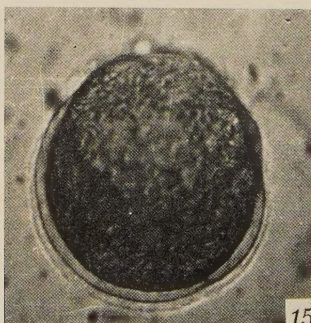
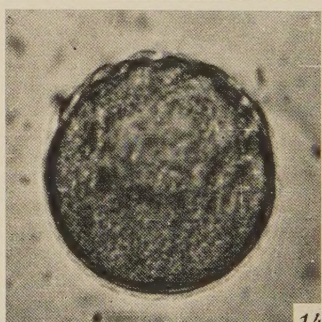
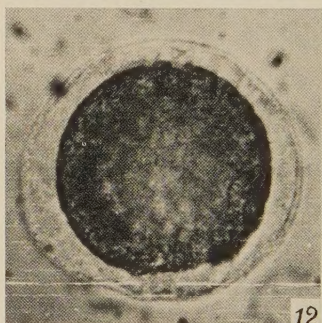
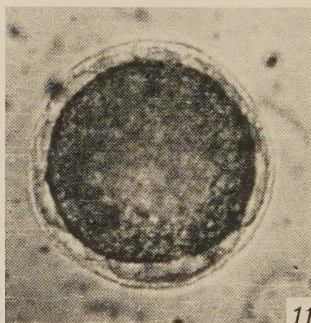
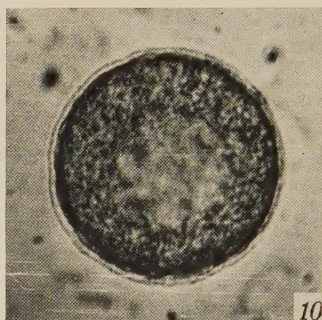
cytolysis: then the condition of their surface was checked by the hypo- and hypertonicity tests. In hypotonic sea water a surface layer appears over the granular endoplasm; then bursts, contracts quickly and breaks down into granules, in the same way as in oocytes pretreated with a proteolytic enzyme. In hypertonic medium the oocytes, treated with bee venom in the same way, present a "vitelline layer" granular and much thinner than the controls.

Treatment in hypertonic medium. The oocytes are first plasmolysed in hypertonic sea water, and then transferred into a 0.005 per cent solution of bee venom in the same hypertonic medium. Within 3 minutes the "vitelline layer" becomes thinner and thinner, and elevates as a membrane on the cell surface (Fig. 10). Meanwhile this surface loses the birefringence acquired

Fig. 3. Oocyte plasmolysed in hypertonic medium.

Fig. 4. Oocyte treated with a 0.01 per cent solution of trypsin and then plasmolysed in hypertonic medium.

Figs. 5-7. Oocyte in 0.001 per cent solution of bee venom: Fig. 5: after 2 minutes 30"; Fig. 6: after 4 minutes 30"; Fig. 7: after 5 minutes.



after the plasmolysis; then it separates from the granular endoplasm, forming first a few flat blisters (Fig. 11), and then, by the merging of these blisters, a sort of membrane, which swells and adheres to that formed by the modified vitelline layer. The endoplasm assumes a coarser granular appearance, but otherwise it remains as a compact mass (looser at the animal pole) separated from the "membranes" by a large hyaline space (Fig. 12). No further changes are evident for a long time. No birefringence is visible in any of such structures.

Combined treatment with trypsin and bee venom

Trypsin and bee venom have to be used separately, because trypsin, even in weak concentration, renders the bee venom inactive towards the oocytes and eggs.

As for the mature eggs, if the bee venom is used first in a sufficient concentration (at least 0.005 per cent), it induces the elevation of a fertilization membrane, upon which the trypsin has no action. If the trypsin is used first (e.g. 3 minutes in a 0.001 per cent solution), the bee venom provokes the cytolysis of the egg, as usual, but without a previous formation of a fertilization membrane. The birefringence of the egg surface, still present after the treatment with trypsin, is soon lost in the bee venom solution.

In the oocytes the combined action of trypsin and bee venom causes a stronger modification on the surface layers than the single enzymes. That can be seen both in normal sea water and in hypertonic medium.

Treatment in normal sea water. The oocytes are treated, e.g., for 3 minutes in a 0.001 per cent solution of bee venom, and then transferred into a 0.1 per cent solution of trypsin. After 25 minutes they are checked by the hypotonicity test. In these conditions the whole oocyte swells, without any evidence of a separate membrane; soon the surface breaks down into granules and cytolysis ensues. After a shorter treatment (e.g. 2 minutes in bee venom and 12 in trypsin) the oocyte surface still elevates as a membrane, that later breaks into granules.

Treatment in hypertonic medium. The oocytes are first treated with trypsin in normal sea water (e.g. 1 hour in a 0.01 per cent solution); then plasmolysed

Figs. 9-12. Oocyte plasmolysed and transferred in a 0.005 per cent solution of bee venom in hypertonic medium: Fig. 9: after 1 minute; Fig. 10: after 3 minutes; Fig. 11: after 5 minutes; Fig. 12: after 7 minutes.

Figs. 13-16. Oocyte treated 4 hours in 0.001 per cent solution of trypsin, plasmolysed and transferred into a 0.003 per cent solution of bee venom; Fig. 13: after 30 seconds; Fig. 14: after 3 minutes; Fig. 16: after 6 minutes 30".

in hypertonic medium, and lastly transferred in a 0.005 per cent solution of bee venom in the same hypertonic medium. Soon after plasmolysis the "vitelline layer", as described above, looks like a "brush border" (Fig. 13), and the underlying surface is birefringent. Within a few minutes this vitelline layer dissolves completely into hyaline granules, agitated by Brownian movement (Fig. 14). The underlying surface, which soon loses its birefringence, elevates in a sort of membrane (Fig. 15), which very quickly breaks down into transparent granules, with Brownian movement. The endoplasm remains in a compact mass, thus surrounded by a halo of hyaline granular material (Fig. 16), not dispersed probably because of the jelly layer.

DISCUSSION

Our conclusions are limited to the *Psammechinus* oocytes and eggs, as differences could exist between the species of sea urchin (cf. 4): however the structure of the surface layers is likely to be fundamentally the same in all species, as their physiological behaviour points out. Another limitation is due to the possible differences between the action of the enzymes on the living cell and that studied *in vitro* (cf. 10): we assume that in both cases the action is similar.

The surface layers of the oocytes and mature eggs of *Psammechinus* are strongly modified by the bee venom, as well as by proteolytic enzymes. The active substance of the bee venom, phospholipase A, acts specifically on lecithins and cephalins, splitting off the unsaturated fatty acid (5, 31). The products of the reaction are lysolecithins and lysocephalins, which are able to change the permeability of many cells (31). The chemical analysis of the lipids in the eggs of *Echinocardium cordatum* made by Öhman (20) has demonstrated the presence of both lecithin (partly bound to proteins) and free cephalin. Such substances are likely to be present in the eggs of *Psammechinus* too and take part in the structure of the surface layers. We do not know if the cytolysis brought forth by the bee venom is due directly to the splitting of phosphatides or indirectly to the lysocithins so formed: Öhman (20) has seen that lysocithins have a strong cytolysing effect on the *Psammechinus* eggs.

The action of bee venom on the oocytes and eggs can be explained by assuming that phospholipids have an important role in the structure of the surface layers, as affirmed by Runnström, Monné, and Broman (28) and by Monroy and Monroy Oddo (19). The phospholipids seem to be responsible for the birefringence of the surface layers, as this birefringence disappears

very easily by action of the bee venom, whereas it is not impaired by proteolytic enzymes. That confirms what already Runnström, Monné, and Broman (28) have observed, i.e. that "agents which bring about a liquefaction of the lipoids of the cortex lowers considerably the birefringence of the cortex". The same effect on the birefringence, caused by bee venom, has been also described by Runnström, Monné, and Wicklund (29). Analogous conclusions have been reached by Monroy (17). A further support to the assumption that this birefringence is due to lipids, may be given by the dichroism which appears after vital staining with Nile blue sulfate. According to Schmidt (30) a dichroism like that described above, is due to lipid lamellae arranged in the radial direction. We have seen that the oocytes and eggs stained with Nile blue sulfate become dichroic only in a hypotonic medium, when their surface layers are in some extent stretched and modified: however it is likely that even in normal conditions lipid lamellae arranged radially are present in the surface layers. It is generally admitted that in the surface layers there are lipid lamellae disposed radially and protein sheets arranged tangentially (for references, cf. (25)), and that the form birefringence due to the lipids masks the intrinsic birefringence (negative radially) of the proteins, cf. also (4). Recently Mitchison and Swann (15) have expressed the opinion that the birefringence may be due only to protein arrangement. We think that it is difficult to explain with such a hypothesis the results obtained and especially the dichroism due to the Nile blue sulfate. The elevation of membranes in both the mature eggs (fertilization membrane) and in the oocytes, as described above, caused by bee venom, could be explained by assuming that the lipid lamellae are covering and compressing a layer of coiled protein molecules, which unfold and stretch when the lipids are broken down: in the lipid lamellae the phospholipids seem to have a key position.

Oocytes and mature eggs present a different degree of sensitivity and a different reaction to proteolytic and bee venom enzymes: already Runnström and Monné (27) have pointed out the striking differences in the structure of their surface. The bee venom proves to be more active on the oocytes and the proteolytic enzymes on the mature eggs. A conclusion that may be drawn is that during maturation there is a decrease of the phospholipid fraction sensitive to the action of phospholipase A. Such a variation in the surface lipids during maturation can bring about a decrease in permeability. In fact, a decrease in permeability to some vital dyes has been described by Runnström (21), and we too in a few experiments with methylene blue, neutral red and Nile blue sulfate observed a deeper and faster

staining in the oocytes than in the mature eggs. Also the different rate of swelling in hypotonic sea water (Fig. 1) can be accounted for a greater permeability in the oocytes. Though the lipid fraction seems to be more important in the oocytes, it has not so regular an arrangement as in the mature eggs: the lipid lamellae cause a birefringence in the oocytes only in hypertonic medium: here probably a narrowing of the interlamellar spaces occurs, as suggested by Monroy (18).

For a further discussion on the changes the surface layers undergo during maturation we must bring forth the question of the separate existence of membranes or layers morphologically distinct. In the oocytes plasmolysed in hypertonic medium a "vitelline layer" becomes evident (Fig. 3). This structure is visible even in the normal oocytes as a thin layer with a bluish tint. It has been also clearly demonstrated by Afzelius (personal communication) with the electron microscope: it is seen as a layer 0.5 to 0.8 micron thick, with a rather loose structure, separated from the rest of the cytoplasm by a fairly even layer or membrane, about 0.1 micron thick. The "vitelline layer" is not present in the mature eggs: here the electron microscope reveals a membrane, composed by a double layer and 120 Å thick, with sparse small pseudopodia not longer than 0.3 micron. Similar observations with the electron microscope have been made by McCulloch (13) in oocytes and eggs of *Arbacia punctulata*.

It is possible that the "vitelline layer" of the oocytes is lost as such during maturation, and that the vitelline membrane of the mature eggs derives from the plasma membrane of the oocytes. As a matter of fact, the "vitelline layer", as seen in hypertonic medium, presents various degrees of compactness and thickness in eggs that have not attained the full cytoplasmic maturation (for such eggs, cf. (27)). Also with the electron microscope the "vitelline layer" is not continuous in oocytes which do not present a germinal vesicle. Furthermore, in hypertonic medium the "vitelline layer" is not birefringent, as the underlying cell surface; this latter has the same type of birefringence as the surface layers of the mature eggs. If the "vitelline layer" is lost as such during maturation, the question arises of its function and of the fate of its materials, composed at least of proteins and phospholipids, as it has been shown. No data are presented on this subject.

It is possible that the plasma membrane of the oocytes and the vitelline membrane of the mature eggs, as well as the "vitelline layer", are structures morphologically distinct from the rest of the cytoplasm. By the action of the bee venom both membranes can separate from the endoplasm, though in different conditions and with different results. Our observations cannot be

conclusive, as in many of our experiments we subjected the surface layers to hypo- and hypertonic mediums, with possible modifications from the normal state. However, evidence of the separate existence of the vitelline membrane in the mature eggs of *Arbacia* can be deduced from the results of Chambers (3) and Kopac (9). Recently Hillier, Lansing, and Rosenthal (8) reported that in *Arbacia* eggs no vitelline membrane is visible with the electron microscope, whereas such structure appears to be present in the electron micrographs made by McCulloch (13). As for *Psammechinus*, there are the mentioned results obtained by Afzelius, where a vitelline membrane is distinguishable.

An electron microscope study of the surface layers as modified by proteolytic and bee venom enzymes is now in progress in collaboration with Afzelius.

SUMMARY

1. Oocytes and mature eggs of *Psammechinus miliaris* have been subjected to the action of proteolytic enzymes (trypsin, chymotrypsin and papain), and of bee venom, whose active factor is a phospholipase A.
2. The surface layers of both oocytes and eggs appear to be very sensitive to such enzymes. The oocytes seem to be more sensitive to bee venom than the mature eggs, and these latter, on the contrary, seem to be more sensitive to proteolytic enzymes than the oocytes.
3. The proteolytic enzymes cause a partial dissolving in the surface of oocytes and eggs. The bee venom induces elevation of a fertilization membrane in the mature eggs. An elevation of both the vitelline layer and of the plasma membrane was observed in oocytes treated with bee venom in hypertonic solution.
4. The birefringence (radially positive) of the mature eggs is not affected by proteolytic enzymes, but it disappears with bee venom. The same applies to the oocytes, which are not birefringent in normal sea water, but become birefringent (positively in the radial direction) in hypertonic solution.
5. The combined action of both proteolytic and bee venom enzymes brings about a complete dissolving of the surface layers.
6. The structure of the surface layers is discussed on the evidence of the experimental results.

I wish to thank Prof. John Runnström for suggesting and directing these investigations. I am also grateful to the authorities of the Kristineberg Zoological Station for the use of their facilities; and to the Swedish Institute for the grant of a scholarship.

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ELECTRON MICROSCOPE STUDY OF NERVE FIBRES GROWN *IN VITRO*

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THE culture of nervous tissue is a very good material for the study of the ultrastructure of nerve fibres. The behaviour of this tissue *in vitro* has been studied thoroughly with the light microscope by G. Levi (3) and collaborators, P. Weiss (12), M. Murray (8) and many others.

De Robertis and Sotelo (1) examined nerve fibres and cells in cultures of embryonic chicken brain with the electron microscope.

Especially good conditions for such an investigation offers the culture of spinal ganglia. Here we have during the first 24 hours of incubation the growth of nerve fibres only. Sometimes they are completely isolated, or they form a complicated network (Figs. 1 and 3). The growth of the spindle cells of the fibroblast type generally begins later. It is even possible to retain these cells for some time (5) lowering the incubation temperature slightly. The nerve fibres grown in these cultures have no collagene (7) and no myeline, since most of them are regenerated after the removal of the ganglion from the embryo. Many of them are thin enough for a good penetration of the electron beam, so that they can be studied without inclusion and sectioning. The nerve cells themselves remain in the centre of the explant (Fig. 2). They do not migrate. If, very seldom, we find some nerve cell, or a small group of them outside the centre, it is because they have been mechanically dislocated during the manipulation. Compared with the spindle cells of the culture, they are very large and thick, so that they can easily be distinguished from the former. Sometimes, fibroblast-like cells adhere to the nerve fibres in the periphery (Fig. 1), and upon superficial observation they might be taken for small nerve cells. However, prolonged observation of these cells showed that their contact with the nerve fibres was not permanent. The examination of the same field, after fixation and impregnation with Cajal's silver method, revealed in hundreds of preparations of the spinal

¹ This investigation has been carried out in the "Instituto de Biofísica da Universidade do Brasil" Rio de Janeiro (Dir. Dr. Carlos Chagas) and has been partly supported by the National Research Council of Brazil.

ganglia as well as of the sympathetic ganglia and embryonic brain (6) that the origin of these fibres in the periphery was in the large nerve cells in the centre of the explant.

MATERIAL AND TECHNIQUE

For the present investigation, spinal ganglia were cultivated on cover slips, which had been coated previously with Formvar 0.3 per cent, and were later transferred, with the technique advised by Porter and Claude (9, 10), to the electron microscope grids. The ganglia were taken from chick embryos of 8–15 days of incubation. They were cultivated—in *toto* or cut in 2–3 fragments—with the Maximow-method in lying drop. Liquid medium was used, consistant of chick serum, Ringer sol. and chick embryo extract in equal parts, or a plasma clot of chick plasma (2), chick embryo extract (2) and Ringer sol. (5).

After 24 to 48 hours of cultivation at 35–37°, the cultures were fixed in osmic acid vapours from 2 to 5 minutes. In order to avoid damage, the fixation was done before the washing or any other manipulation of the cultures, very often when still in the interior of the incubator. This precaution proved especially useful for the cultures in liquid media, where the adhesion of the cultures to the film is fragile, and a slight movement could detach them from the film. Only after the fixation were the cultures washed in distilled water. With the help of a binocular dissecting microscope, the central part of the culture was then removed with a very fine forceps in a rapid grip. With this operation the nerve fibres, which are already fixed, rupture at the margin of the explant, but do not detach any more from the film. In the cultures which were cultivated in a clot, the latter was removed only after having removed the central portion. Also in these cultures, some fibres are always attached to the film.

The interesting regions were then transferred, after control in the light microscope, to the electron microscope grids, and were later shadowed with chromium. The shadowed preparations were then observed in the electron microscope "Philips" with 40, 60, and 80 KV.

OBSERVATIONS

Generally, in these preparations, we see isolated nerve fibres with their terminal extensions (Fig. 5 a). Besides the isolated fibres, there are nets of

Fig. 1. Spinal gangl. of chick embryo. Growth area with nerve fibres which are, in the proximal region, covered by spindle cells. Phase contrast photogr. of living culture.

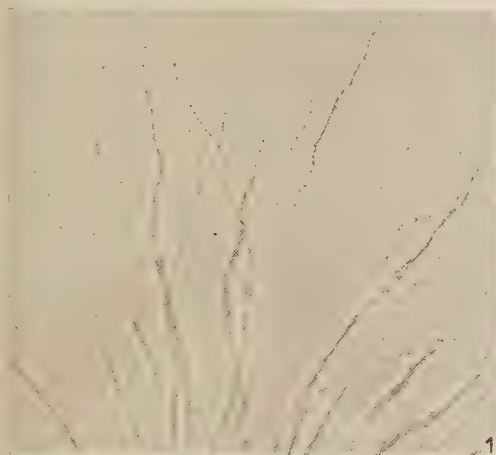
Fig. 2. Same culture as Fig. 1. Central region with large nerve cells. Phase contrast photogr. of living culture.

Fig. 3. Same culture as Figs. 1 and 2. Growing ends of nerve fibres. Phase contrast photogr. of living culture.

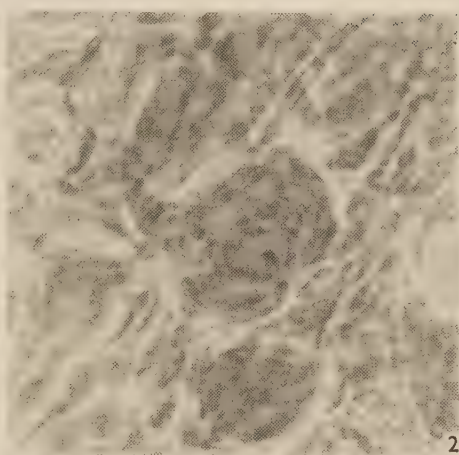
Fig. 4. Culture 10.017. Spinal gangl. of 14 days chick embryo in liquid medium. Two nerve fibres running over fibroblast, Magn. 4 300 ×.

Fig. 5a. Culture 10.394. Spinal gangl. of 9 days chick embryo in plasma clot. Terminal extension of growing nerve fibre. Magn. 2 200 ×.

Fig. 5b. Higher magnification of rectangular region in 5a. Magn. 15 600 ×.



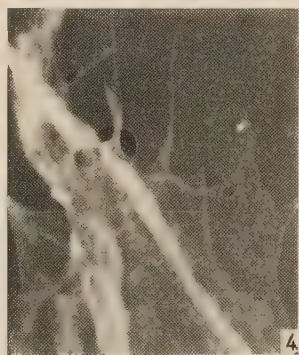
1



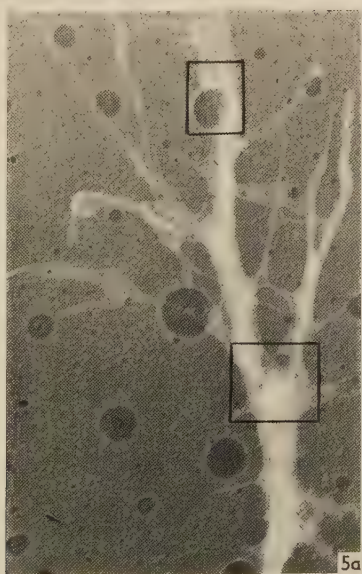
2



3



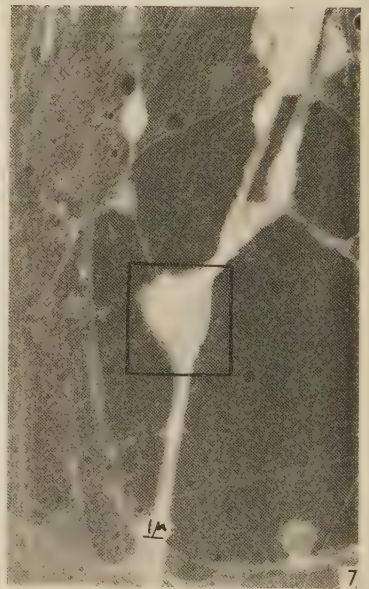
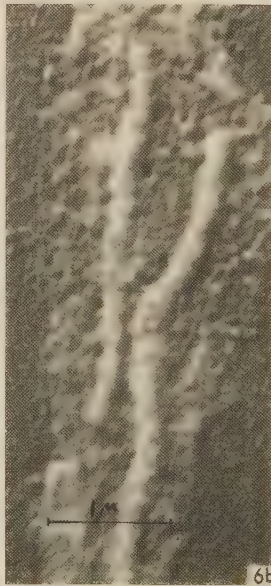
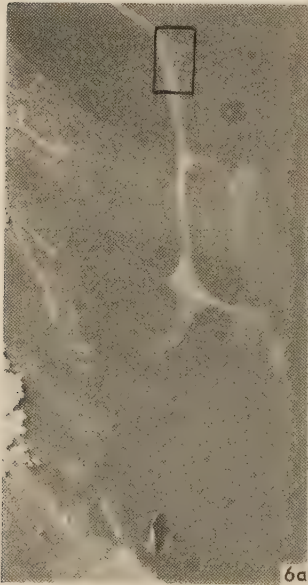
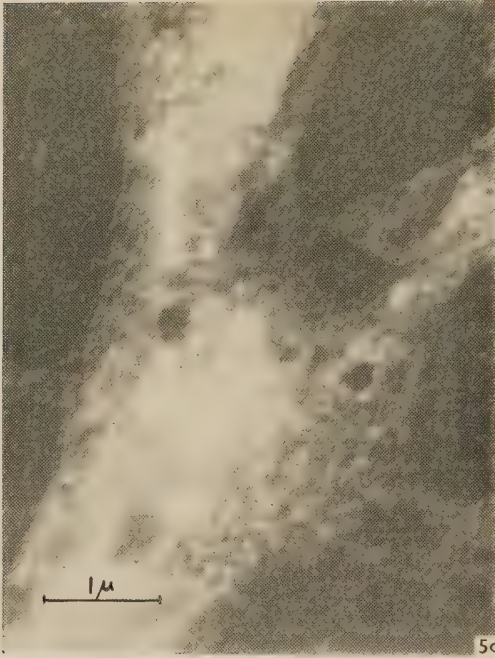
4



5a



5b



anastomosed fibres, and often also some cells of the fibroblast type, which are overrun by nerve fibres (Fig. 4, 6 a). The great observation field of the "Philips" microscope, and the ease with which one passes from a very low to a very high magnification, made it possible to control and to record the course of the nerve fibres over a great area, and to study their relations to the cells in the periphery. None of these cells are nerve cells, the nerve fibres run over them, and it is easy to follow them up to the point of their rupture at the margin of the removed explant.

The nerve fibres of medium size and the larger ones show, already with lower magnifications, a longitudinal striation. With higher magnification it can be seen that their cytoplasm is composed of fine fibrils which run parallel and show a regular discontinuity. This discontinuity is more pronounced when the nerve fibres are large and their fibrils abundant. In these instances, the fibrils seem to be compressed, but it was not possible to detect any membrane which would keep them together. The regular discontinuity goes over directly into a more beady appearance in those points where the fibre spreads in surface. The exact diametre of the fibrils is difficult to establish because of the more or less pronounced spreading of the cytoplasm. It varies from 250 Å to 400 Å. More seldom, thin, isolated fibres are seen which may be perfectly smooth, or show the same discontinuity of the fibrils in the cytoplasm; and small fibres are also found, in which the longitudinal striation is smooth.

In many fibres the longitudinal striation can be followed up to their terminal extensions (Fig. 5 b, c). These terminal extensions are much more complex than it is possible to see in the light microscope. Besides the amoeboid membranes—well known since the observations of Harrison—very fine and long prolongations can be observed which anastomose between each other, or with the own fibre, or with other fibres in the neighbourhood. Sometimes one fibre, after a first extension will continue as a fibre for some time and extend again. Not always the striation is visible in them. They may have a more granular or spongy appearance, due possibly to overlaying of membranes. Often we find round holes in the centre of these extensions and also, especially when they are very thin, inclusions in form of filaments,

Fig. 5c. Higher magnification of rectangular region in Fig. 5a. Magn. 15 600 ×.

Fig. 6a. Culture 10.373. Spinal gangl. of 11 days chick embryo in plasma clot. Terminal extension of growing nerve fibre with mitochondria-like inclusions. Magn. 1 200 ×.

Fig. 6b. Higher magnification of rectangular region in Fig. 6a. Magn. 16 000 ×.

Fig. 7. Culture 10.394. Spinal gangl. of 9 days chick embryo in liquid medium. Enlargement in nerve fibre, fine longitudinal striation in cytoplasm preserved. Magn. 16 000 ×.

which recall mitochondria of other cells (Fig. 6 a, b). Sometimes groups of these filaments are observed in those points where the nerve fibre spreads to form the terminal extension.

The large nerve fibres as well as the fine ones may have ovoid or fusiform enlargements. In the large fibres, these formations occur often where two fibres meet. The longitudinal striation in them may persist (Fig. 7). There can also be observed a transversal striation which is probably due to the periodicity of the fibrils. In the enlargements of the smaller fibres there is no definite structure to be seen, they may appear transparent or cloudy, often they give origin to new lateral prolongations.

DISCUSSION

The structures described above of nerve fibres grown *in vitro* are similar to those which have been obtained in ultrafine sections of peripheral nerves by Schmitt (11), Fernández-Morán (2) and others, and by Fernández-Morán from the very thin unmyelinated nerve fibres of the central nervous system of various animals. All these authors describe the axoplasm as composed of very thin fibrils, which have a parallel arrangement and show a certain regular nodosity. The diameter of these fibrils varies from 100–250 Å. The difference in diameter between the fibrils of these authors and those obtained in tissue cultures is probably due to the different technique.

The tissue culture has the advantage that here the technical damages may be reduced to a minimum. It is not necessary to make inclusions and ultrafine sections. Fixing the cultures still in the incubator without washing them or giving them any other treatment, no alterations are produced in the fibres which are postmortal or due to lesions during the manipulations. The obtained images are of fibres which are, at the moment of fixation, in perfect state and growing actively. The observation in the light microscope showed that the fibres, after fixation with osmic acid vapours, had the same aspect as before the fixation, but that they had somewhat shrunk, their surface was not so smooth as it had been before, after having dried on the electron microscope grids. This shrinking of the fibres on the grids is certainly very slight compared with what happens during the histologic processes of inclusion, freezing etc. We also have to consider that cells and fibres in tissue cultures spread very much in surface, which would easily give values above the normal. In the terminal extension of the nerve fibre in Fig. 5, for instance, the fibrils in Fig. 5 c are finer than those in Fig. 5 b where the spreading is greater.

The micrographs which Fernández-Morán published of the fine unmyelinated fibres in the central nervous system give the impression that the nerve fibres are composed of a number of independent fibrils which form a bundle by simple adhesion. No ground substance or matrix seems to be present. The author himself considers the possibility of a loss of some liquid or other substance during the processes of preparation.

In the tissue cultures of spinal ganglia, the conditions are extremely simple. In Cajal preparations of these cultures it has been seen that, and it can be seen at any moment again, from every one of the large nerve cells in the centre of the explant one or two fibres grow out into the periphery, branching repeatedly before they reach the margin of the culture (4). Every terminal extension of the growing fibre can easily be traced back to its point of origin in the nerve cells in the centre. There is no doubt about the existence of fibrils along the whole length of the nerve fibres grown *in vitro*. But the fact that these fibres represent the finest and most distal prolongations of the nerve cell body, and that they act in their terminal extensions as units, forming membranes and having even inclusions and vacuole like formations, give the impression that the striations represent structural units rather than independent fibrils. That is to say that they are structural units of the cytoplasm of the nerve fibre, comparable to those striations which have been found in other proteic tissues.

Whether the inclusions of the cytoplasm in the terminal extensions represent mitochondria, and which is their part in the growth process of the fibres, ought to be studied more thoroughly. Some authors consider that there are mitochondria in nerve fibres and they refer to inclusions which stain intensely in black by the osmic acid, in certain points of the fibres. De Robertis and Sotelo also report the existence of mitochondria in the cultured nerve fibres, however, without giving any demonstration.

De Robertis and Sotelo examined nerve fibres grown *in vitro* of embryonic chicken brain and also observed a longitudinal striation in the fibres. They report the presence of neurofibrils in the cytoplasm of neuroblasts, at the side of mitochondria and microvesicles, which would be the first stages of fibrillogenese in the neuroblasts. Their micrographs of the neurofibrils already formed in the cytoplasm strongly recall the image of Fig. 4 obtained during this investigation, where nerve fibres run over cells of the fibroblast type. The distribution of neurofibrils in the nerve cells, as revealed by the images of Cajal stained preparations, have a different pattern. In the embryonic brain still more than in the spinal ganglion of the chick, there are many different types of cells, which are not nerve cells, and these are

the cells which migrate into the periphery in the first hours of cultivation. Therefore the images obtained by De Robertis and Sotelo ought to be controlled previously or parallel with the classic method of Cajal, in order to be sure about the nature of the cells in observation, and to avoid mistakes in the interpretation of their structure.

The ovoid and fusiform enlargements which are seen in the nerve fibres are well known in tissue cultures. They may appear as an immediate reaction to lesions, but are not necessarily degeneration formations. Systematic experiments are in course for studying in more detail the ultrastructure of these formations and their signification. An exact analysis of the points of anastomosis of two fibres must also be postponed.

SUMMARY

Nerve fibres of spinal ganglia of chick embryos, grown *in vitro*, have been examined in the electron microscope.

A fine longitudinal striation has been observed in their cytoplasm which reaches up to the growing ends of the fibres. The fibrils which are responsible for the striation show parallel arrangement and have a diameter of 250–400 Å. They show a regular periodicity which gradually goes over into a beady appearance, where the fibre spreads, and even fine, smooth fibrils may be found.

The terminal extensions of the growing nerve fibres are more complex than can be seen in the light microscope. Besides amoeboid membranes, numerous fine prolongations are present. Sometimes inclusions can be found in them which are similar to mitochondria, and empty spherical spaces, recalling vacuoles.

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CAROTENOIDS IN THE DEVELOPING EGGS OF THE SEA URCHIN *PARACENTROTUS LIVIDUS*

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THE problem of the function and metabolism of carotenoids in marine invertebrates is one that is essentially unsolved although it has been tackled on a number of occasions (see Goodwin (3) for a review). Recently Monroy, Monroy-Oddo and de Nicola (13) reported the results of an investigation which indicated that during the early stages of development of fertilized *Paracentrotus lividus* eggs, the carotenoids originally present tended to disappear, suggesting a metabolic function during embryogenesis. Monroy and de Nicola (14) also found that the pigments occur attached to proteins in the pigment granules of the eggs, again suggesting a metabolic function (3).

The first intensive investigation into the nature of the pigments of the gonads of *P. lividus* was carried out by Lederer (9, 10). He found α - and β -carotene to be present in small amounts and also isolated for the first time the keto-carotenoid echinenone and the highly oxygenated pentaxanthin. He also noted in small amounts the presence of a compound similar to fucoxanthin, but separable from it chromatographically, and a further unidentified xanthophyll. Recently de Nicola and Monroy-Oddo (12) have reported briefly on the differential distribution of the carotenoids in the eggs, ovaries and testes of *P. lividus*.

The present investigation is concerned with a more detailed study of the carotenoids of the gonads of *P. lividus*, together with observations on the distribution of the pigments in developing eggs.

EXPERIMENTAL

Materials. The specimens of *P. lividus* examined were collected in the Bay of Naples and kept in the laboratory in sea water tanks until required.

Extraction of pigments. The ovaries and testes were ground to a fine dry powder with anhydrous Na_2SO_4 and extracted in the cold with successive portions of ethyl ether until all the pigment had been removed. The ether extracts were then combined, dried over anhydrous Na_2SO_4 and taken to dryness at room temp. under N_2 .

The suspended eggs were centrifuged and the resulting egg mass treated in one of

two ways:— (a) as just described for ovaries and testes or (b) according to the method of Monroy *et al.* (13), in which the eggs are shaken with a 7:3 mixture of ethanol and light petroleum. Most of the pigment passes into the light petroleum which can be collected in a separatory funnel. The remaining pigment can be extracted from the ethanol layer by adding a small vol. of ether and then cautiously adding water dropwise until two layers form, leaving the pigment in the ether layer. The combined ether-light petroleum extracts are then washed free from ethanol and then treated in the usual way.

Saponification. Saponification of the pigment extracts was carried out by the method of Goodwin and Srisukh (6), either at 30–40° or at room temp.

Chromatography. The pigment extracts were separated chromatographically using weakened alumina as adsorbent and light petroleum containing varying amounts of ethyl ether as developer (6).

Quantitative determination of pigments. This was carried out by dissolving the various fractions in a known vol. of light petroleum and measuring the extinctions (E) at the wave lengths of maximum absorption on a Beckman photoelectric spectrophotometer. The $E_{1\text{ cm}}^{1\text{ per cent}}$ (λ max.) values used for known pigments are those given by Goodwin (8); when the pigments under examination have not previously been reported upon, the $E_{1\text{ cm}}^{1\text{ per cent}}$ (λ max.) value has been assumed to be 2500.

Fertilization experiments. These were carried out at 20° under constant stirring as previously described by Monroy *et al.* (13).

Nitrogen determinations. These were carried out on the eggs using the micro technique of Kjeldahl.

RESULTS

Eggs. Chromatography of the pigment extract of freshly shed unfertilized eggs gave the separation recorded in Table I. Fraction A_1 contains very small amounts of β -carotene. A_2 contains mainly echinenone, identified by its single banded absorption spectrum with λ max. at 452–3 $m\mu$ in light petroleum, its adsorptive power and its epiphasic behaviour when partitioned between light petroleum and 90 per cent (v/v) aqueous methanol. As earlier noted by Lederer (9), however, there also exist small amounts of a pigment very similar in all its properties to echinenone; it may possibly be a *cis*-isomer of echinenone. Fractions A_3 and A_5 represent closely related red pigments which have quite unique properties; their absorption spectra are recorded in Fig. 1. On saponifying both these pigments they immediately change colour from red to orange-yellow, and the resulting pigments, which are extractable from the saponified solution with ether, then exhibit the very characteristic three banded absorption spectra of non-ketogenic carotenoids (Fig. 1). Further experiments showed that if a few drops of alkali (KOH) are added in the cold to an ethanolic solution of either A_3 or A_5 , the colour change occurs instantaneously, no heat being required; neutralization of the yellow solution does not restore the red colouration. Furthermore, when

TABLE I

Chromatographic separation of the pigments of unfertilized eggs of *P. lividus*.

[Adsorbent: weakened alumina; developer: light petroleum containing varying amounts of diethyl ether and/or ethanol; pigments in order of increasing adsorptive power.]

Zone No.	Description	Absorption maxima (m μ) in light petroleum	Concn. of ether in light petroleum required to elute zone	Identification
A ₁	yellow; trace	425, 448, 473		β -carotene
A ₂	orange-red	453-4		echinenone ¹
A ₃	red	488-490	20-40	new pigment paracentrotin A (= xanthophyll monoester of de Nicola and Monroy-Oddo)
A ₄	yellow; trace		50	probably = xanthophyll diester of de Nicola and Monroy-Oddo
A ₅	pink	480	100	new pigment, paracentrotin B (= xanthophyll 1 of de Nicola and Monroy-Oddo)
A ₆	yellow	455-6	100 + 2 per cent ethanol	(? hydroxy derivative of echinenone (= xanthophyll 2 of de Nicola and Monroy-Oddo)
A ₇	strong adsorbed residue	Flat 440-450	100 + 25 per cent ethanol	probably a mixture of polyhydroxy xanthophylls (= partly xanthophyll 6 of de Nicola and Monroy-Oddo)

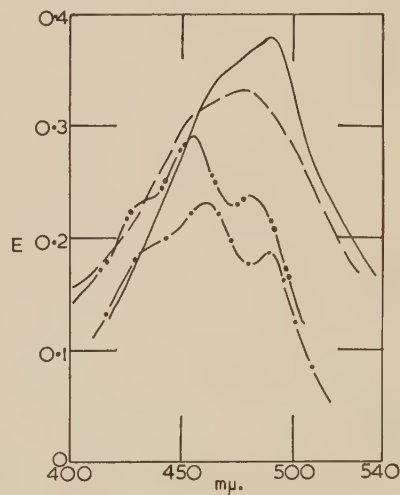
¹ This echinenone fraction contains traces of a second, very similar pigment.

Fig. 1. The absorption spectra in light petroleum of paracentrotins A and B.

— Paracentrotin A;
 - - - Paracentrotin B;
 . . . Paracentrotin A after treating with alkali;
 - . . Paracentrotin B after treatment with alkali.

a solution of either of these pigments in light petroleum is shaken with a 10 per cent (w/v) Na_2CO_3 solution no colour change takes place nor is the pigment extracted into the alkaline phase, as would be expected if it were an acidic carotenoid.

After the saponification process the new pigments do not differ from their precursors to any great extent in adsorptive power or in their reaction in the partition test. This suggests that the changes brought about by saponification does not in either case include the release of a free hydroxyl group and the pigments, therefore, may not be xanthophyll esters in the normal sense of the term, as suggested by de Nicola and Monroy-Oddo (12).

These pigments are so completely unique in the carotenoid field that it is proposed to give them the names paracentrotin A and B respectively, although they have not yet been obtained in crystalline form. This nomenclature fits in with the general recommendations on carotenoid nomenclature (see Karrer (8)) which suggests that if there is doubt about the nature of a carotenoid it should be given an arbitrary name, preferably one ending in 'in'. Similarly the pigments into which paracentrotins A and B are converted are not identifiable with any known carotenoids.

Fraction A_6 from its rather flat single banded spectrum and its high adsorptive power may be an hydroxy keto-carotenoid, perhaps an hydroxy derivative of echinenone. A_7 represents a very strongly adsorbed xanthophyll-fraction and is probably a mixture.

Testes. The chromatographic separation of the unsaponified pigments from the testes of *P. lividus* is given in Table II. It will be seen that echinenone is by far the predominant pigment, but that small traces of hydroxylated xanthophylls do occur; one is somewhat similar to lutein and the other similar to Lederer's pentaxanthin.

The separation of the pigments of the testes was also examined after a preliminary saponification, with exactly similar results to those obtained with the non-saponified extract.

Ovaries. The pigments of the spent ovaries have been examined in the usual way and the resulting chromatogram is described in Table III. It will be seen that, together with β -carotene and echinenone, paracentrotins A and B are also present as well as small amounts of hydroxy-xanthophylls. Of the xanthophylls C_5 and C_9 have not been identified; C_6 is the same pigment as A_6 (Table I), found in the eggs, C_7 as B_4 (Table II) and C_8 is probably pentaxanthin. Saponification of the ovarian pigments before extraction produced the usual changes in paracentrotins A and B, but did not affect the remaining pigments to any appreciable extent.

TABLE II

Chromatographic separation of the pigments of testes of *P. lividus*.

[Adsorbent weakened alumina; developer light petroleum containing varying amounts of diethyl ether and/or ethanol; pigments in order of increasing adsorptive power.]

Zone No.	Description	Absorption maxima (m μ) in light petroleum	Concn. of ether (% v/v) required to elute zone	Identification
B ₁	yellow	425, 448, 473	1	β -carotene
B ₂	orange red	453	8-15	echinenone ¹
B ₃	yellow diffuse	420, 445, 470	80	may be lutein (= xanthophyll 3 of de Nicola and Monroy-Oddo)
B ₄	trace of yellow	ill defined band at 450	100	—
B ₅	yellow	420, 444, 470	100 + 10-20 per cent ethanol	probably pentaxanthin

Quantitative distribution of pigments in gonadal tissues. De Nicola and Monroy-Oddo (12) have previously demonstrated the differential distribution of carotenoids in eggs, testes and ovaries. Experiments carried out during this investigation have fully confirmed their findings, as will be seen from the results set out in Table IV. The carotenoids in the testes consist almost entirely of β -carotene and echinenone, paracentrotins A and B being completely absent. Eggs on the other hand contain considerable amounts of these two pigments, much less β -carotene and echinenone and about 20 per cent of an unidentified xanthophyll. Ovaries tend to fall between the testes and eggs, containing some paracentrotins A and B as well as considerable amounts of echinenone and β -carotene. It is possible that using ovaries completely freed from eggs, it would be found that they did not accumulate any paracentrotins.

Pigment distribution in developing eggs. Experiments were carried out in order to determine the quantitative and qualitative changes, if any, in the carotenoids of developing *P. lividus* eggs. Eggs were examined unfertilized, at the free-swimming blastulae stage and at the plutei stage. The chromatographic analyses showed that there were no significant changes either in the relative distribution or in the type of pigments present during these stages

¹ Also contains traces of a second pigment very similar to echinenone.

TABLE III

Chromatographic separation of the pigments of the spent ovaries of *P. lividus*.

[Adsorbent, weakened alumina; developer light petroleum containing varying amounts of diethyl ether and/or ethanol; pigments in order of increasing adsorptive power.]

Zone No.	Description	Absorption maxima (m μ) in light petroleum	Concn. of ether (per cent v/v) required to elute zone	Identification
C ₁	trace of yellow	448, 472	0	probably β -carotene
C ₂	orange brown	453	8-16	echinenone
C ₃	pinkish	462, 488	50-70 per cent	{ probably paracentrotin A and B, respectively
C ₄	purple	465, 490	100 per cent	
C ₅	yellow	455, 480	100+1 per cent ethanol	?
C ₆	brownish yellow	459.6 flat	100+2 per cent ethanol	= A ₆
C ₇	brownish	450	100+4 per cent ethanol	= B ₄
C ₈	brownish	442, 469	100+25 per cent ethanol	probably pentaxanthin
C ₉	pink very strongly adsorbed	450 (single band)	removed only with glacial acetic acid	?

TABLE IV

The relative distribution of carotenoids in gonadal tissues of *P. lividus*.

Pigment	Percentage of total pigments present		
	Testes	Ovaries	Eggs
β -Carotene	10.0	9.8	3.7
Echinenone	85.6	50.2	24.1
Paracentrotin A	0	9.8	30.9
A ₄ (Table I)	0	—	trace
Paracentrotin B	0	2.8	11.0
C ₅ (Table III)	0	5.0	0
A ₆ (Table I)	0	3.5	20.1
B ₃ (Table II)	2.3	0	0
B ₄	0.7	4.4	0
Pentaxanthin (?)	0	1.5	0
Other strongly adsorbed xanthophylls	1.4	5.0	10.2

of development (see Table V). The single slightly anomalous result, increased paracentrotin B and decreased A₆ in the blastula stage, is not significant because in the later plutei stage it returned to its original value. It is probably due to some A₆ contaminating the paracentrotin B fraction.

TABLE V

The relative distribution of pigments in developing *P. lividus* eggs.

Pigment	Per cent of total pigment present		
	Unfertilized eggs	Free swimming blastulae (24 hrs. old)	Plutei (72 hrs.)
β-Carotene	3.7	3.8	4.3
Echinenone	24.1	24.5	22.0
Paracentrotin A	30.9	28.5	26.8
Paracentrotin B	11.0	21.9	14.1
A ₆ (Table I)	20.1	10.0	18.4
Other strongly adsorbed xanthophylls (including pentaxanthin)	10.2	11.3	14.4

It was noticed that at the plutei stage, the residue obtained after removal of the carotenoids was still coloured. The pigment responsible could be extracted with ether only after addition of dilute HCl; it was only present in minute amounts and could not be examined further, but it is probably an echinochrome type of pigment. This pigment has already been noted by Monroy *et al.* (13).

Concentration of carotenoid in eggs. A small number of experiments were carried out to determine the total amount of pigments in the eggs (referred to total N of the eggs), in order to see if the observations of Monroy *et al.* (13), that there is a decrease in pigments during the early stages of embryogenesis, could be confirmed. The results were, however, somewhat variable and no clear cut picture emerged. It was found, however that the mean total carotenoid content of *P. lividus* eggs was of the order of 10–20 µg/mg total N.

DISCUSSION

The results reported here amply confirm the original observations of de Nicola and Monroy-Oddo (10), that there is a differential distribution of carotenoids in the gonads of *P. lividus*. The most important differentiation

being the existence in the eggs, and to a lesser extent in the ovaries, of paracentrotins A and B. In this respect *P. lividus* differs considerably from the only other echinoid examined in detail, viz.: *Sphaerechinus granularis* (11). In this sea urchin there was no suggestion of a differential carotenoid distribution. In *Lytechinus pictus*, however, there may be some differentiation for it has been found that the ratio of xanthophylls to carotenes varies between the gonads and testes (6).

Furthermore, no differentiation in the gonads of the only other marine invertebrates in which males and females have been examined separately has been noted, viz.: *Strongylocentrotus purpuratus* (11), *Patella vulgata* (2) and *P. depressa* (2). Full details of carotenoid distribution in other marine invertebrates can be obtained from the recent monographs of Goodwin (5) and of Fox (1).

The interesting problem arises as to whether paracentrotins A and B are functional in the eggs. If utilization of the pigments is taken as a criterion of function then Table V quite clearly shows that during early development to the plutei stage there is no specific utilization of these two pigments compared with the other pigments present. According to Monroy *et al.* (13) however, there may be a general utilization of all the pigments during these early development stages.

The problem of the structure of the two new pigments paracentrotins A and B is a very real one, for their properties are quite unique and no structure can be envisaged, arguing from analogy with known pigments. The elucidation awaits a full-scale attack by organic chemists.

SUMMARY

1. The pigments in the eggs, ovaries and testes of *P. lividus* have been characterized and their distribution determined quantitatively.
2. Two new pigments paracentrotins A and B have been found in the eggs; and to a lesser extent in the ovaries, but not in the testes.
3. There are no significant changes in the relative amounts of the pigments present in developing eggs.

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ON THE ASSOCIATION AND DISSOCIATION OF "PENTOSE NUCLEOPROTEIN" FROM PARTICULATE STRUCTURES OF THE UNFERTILIZED SEA URCHIN EGG¹

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EARLIER investigations reported by Brachet (1) suggested the presence of large amounts of pentose nucleic acid (PNA) and a relatively limited quantity of desoxypentose nucleic acid (DNA) within the unfertilized *Arbacia* egg. These results were subsequently confirmed by Schmidt, Hecht and Thannhauser (17) who reported the nucleic acid phosphorus content of the average unfertilized *Arbacia* egg to be 2.0×10^{-6} μ g PNA-P and $0.07-0.1 \times 10^{-6}$ μ g DNA-P respectively. Elson and Chargaff (4) using a microbiological assay for thymine estimated the DNA content of unfertilized *Arbacia* egg to be approximately 24×10^{-6} μ g per egg. Lansing and Rosenthal (13) using staining techniques found that PNA was localized on the surface of the egg.

The present report will summarize the results of experiments demonstrating the localization of PNA within submicroscopic particulate structures isolated from homogenates of unfertilized *Arbacia* eggs. The report will also include information relative to the nature of binding forces responsible for the association of PNA (presumably as nucleoprotein) with sedimentable particles.

EXPERIMENTAL

Materials and Methods

Preparation of eggs. Urchins were forced to shed their eggs by injecting isotonic KCl (0.5 ml). The shed eggs were thoroughly washed with filtered sea water and packed by light centrifugation (5 minutes at $500 \times g$). The packed eggs were homogenized in appropriate medium (usually 1:10 dilution unless otherwise specified) by repeated forcing through a syringe fitted with a number 20 hypodermic needle (12). All prepared homogenates were checked microscopically to insure the absence of a significant percentage of original whole cells.

¹ An abstract covering part of this work has already appeared (22).

² Lalor Fellow, summer of 1953.

Centrifugation. All low speed centrifugations were carried out in an International Centrifuge. The Sorvall SS-1 and the Spinco Model L Ultra Centrifuge were employed for the higher centrifugal forces.

Chemical analysis. All prepared egg fractions were extracted 2 or more times with cold 5 per cent trichloroacetic acid (TCA) followed by repeated extraction with cold 95 per cent ethanol containing a few drops of TCA, until extraction of echinochrome was complete. The egg fractions were next extracted 3 times in boiling absolute ethanol. With most fractions nucleic acids were subsequently extracted directly by hot TCA using slight modifications (21) of the Schneider method (19). In those fractions requiring a separation of PNA and DNA, extraction procedures described by Schmidt and Thannhauser (18) with appropriate modification (17) were substituted. In most experiments, however, no attempt was made to separate the extremely small amounts of DNA present within the egg fractions. In these experiments all hot TCA extracted nucleic acid was considered to be PNA. Nucleic acids were determined by quantitative ultraviolet absorption methods (21) which were tested in preliminary experiments and found to be applicable for the present material (see Results). Attempts to determine pentose quantitatively were complicated by the presence of other interfering carbohydrates within the PNA fractions and were therefore abandoned. Phosphorus was determined on certain nucleic acid fractions by the method of Fiske-SubbaRow (5).

Protein was estimated on the residues resulting from cold TCA, cold and hot ethanol and hot TCA extraction of the egg fractions unless otherwise specified. Protein was estimated on the basis of nitrogen measurements. Digestions were carried out using methods described by Ma and Zuazaga (14). Ammonia was distilled under reduced pressure by a method described by Speck (20) and determined colorimetrically using a Nessler's Reagent described by Vanselow (23). Tyrosine was measured on a number of the protein fractions using the phenol Reagent of Folin and Ciocalteu (6) and was found to be both convenient and reliable for estimating protein concentrations within the separated egg fractions (see Table I).

TABLE I

Comparative Nitrogen and Tyrosine Content of Protein from Centrifuged Fractions of Homogenized Eggs.

Fraction ¹	Centrifugation		Analyses of Protein Fractions ²		
	Force	Time	Nitrogen	Tyrosine	Ratio N/Tyr.
Initial homogenate . .	—	—	17.7 mg	5.0 mg	3.5
Sup. 1	350 × g	30 min.	11.1 „	3.2 „	3.5
Sup. 2	10 ³ × g	30 „	9.7 „	2.6 „	3.7
Sup. 3	10 ⁴ × g	30 „	7.6 „	2.0 „	3.8
Sed. 4	10 ⁵ × g	30 „	1.9 „	0.53 „	3.6
Sup. 4			6.0 „	1.54 „	3.9

¹ Eggs homogenized in molar KCl homogenate subjected to successive centrifugation and aliquots of the resulting supernates as well as final sediment taken for the protein analyses.

² Results computed for 1 gram original wet weight of eggs.

RESULTS

PNA, DNA and Protein N content of unfertilized whole Arbacia eggs

Preliminary to the centrifugation experiments, whole *Arbacia* eggs were analysed for nucleic acids and protein content (see Table II). The results for nucleic acids are of the same order of magnitude as those reported by others (2, 17, 24). The results for DNA were not exceedingly reliable in view of the limited quantities present in the available material. The ratio of PNA to protein N appears to be similar to that observed for many mammalian tissues.

TABLE II

PNA, DNA and Protein N Content of Whole Unfertilized *Arbacia* Eggs.

Sample	MG PNA/Gm wet ¹		MG DNA/Gm wet ²		MG Protein N/Gm wet ³
	By U.V.	By P	By U.V.	By P	
1	8.2	8.1	0.40	0.23	19.0
2	7.3	7.1	0.36	0.20	17.1

*The distribution of PNA within centrifugally separated fractions
of homogenized eggs*

Experiments using non-ionic homogenizing media. The use of non-ionic homogenizing media was considered to be the most appropriate for the study of nucleic acid distribution for reasons which will be discussed in a later section of this report. In Table III are summarized the results of an experiment demonstrating the relative distribution of PNA within egg fractions obtained by successive centrifugation of a homogenate prepared in molar sucrose over a range of 350—100,000 × g. Protein N was also determined on each of the separated fractions (see Table III). The selected centrifugation times and forces employed are of a somewhat arbitrary nature and do not imply separations into cytologically homogeneous fractions. From the summarized results it can be seen that less than 20 per

¹ PNA extracted by the method of Schmidt-Thannhauser (18) and calculated on the basis of an extinction coefficient ($E_1^{1\text{ per cent}}$) of 340 and P composition of 9.65 per cent.

² DNA extracted (following PNA separation) with hot TCA and calculated on the basis of an extinction coefficient ($E_1^{1\text{ per cent}}$) of 310 and P composition of 10.0 per cent.

³ Protein N determined on aliquots of the dissolved residues resulting from cold TCA and ethanol extractions prior to nucleic acid separations.

cent of the egg PNA is present in particulates sedimented at forces up to $25,000 \times g$. The protein figures show approximately 50 per cent sedimentation within these fractions. The supernate resulting from the $25,000 \times g$ spin was divided into 4 equal volumes and placed in separate plastic tubes. To each of 2 tubes was added an equal volume of molar sucrose (tubes a, b), to the remaining 2 tubes was added an equal volume of water (tubes c, d) for purposes of decreasing density and viscosity of the medium in order to facilitate sedimentation. Spinning at $100,000 \times g$ for a period up to 3 hours showed a continuous sedimentation of PNA such that in 0.5 M sucrose only 5 per cent remained non-sedimented. No attempt was made to follow the DNA in fractions other than the first sediment in which less than 50 per cent of the original appeared to be present.

Appreciable lysis of echinochrome granules was apparent in the molar sucrose homogenates. A maximum stabilization of these granules was achieved only in concentrations of sucrose approaching 2 molar. Eggs

TABLE III

Distribution of PNA and Protein N Within Centrifugally Separated Fractions of *Arbacia* Eggs Homogenized in Molar Sucrose.

Fraction ¹	Centrifugation			Conc./Gm Original Wet Wt of Eggs			
	Force	Time	Sucrose Conc.	PNA	Per cent of Total	Protein N	Per cent of Total
Initial homogenate	—	—	1.0 M	8.0 mg	100	17.2 mg	100
Sediment 1 . .	$350 \times g$	30 min.	„	0.20	2.5	2.5	14
„ 2 . .	$1\,500 \times g$	30 „	„	0.33	4.1	1.1	6.4
„ 3 . .	$25\,000 \times g$	30 „	„	0.87	11	4.4	26
a) Sediment 4a	$10^5 \times g$	60 „	„	1.8	22	0.83	4.8
Supernate 4a				4.6	58	7.7	45
b) Sediment 4b	$10^5 \times g$	180 „	„	4.3	54	1.4	8.1
Supernate 4b				2.0	25	6.9	40
c) Sediment 4c	$10^5 \times g$	60 „	0.5	4.4	55	1.7	9.9
Supernate 4c				2.0	25	6.9	40
d) Sediment 4d	$10^5 \times g$	180 „	0.5	5.9	74	2.1	12
Supernate 4d				0.41	5.1	6.3	37

¹ For further description of fractionation procedure, see text. All sedimented fractions treated directly without further washing for PNA and Protein N analyses.

homogenized in hypotonic sucrose showed considerably more lysis of echinochrome granules than in molar sucrose; however, the extent of lysis did not appear to affect the distribution of PNA (compare with results presented in later sections).

Identical experiments were carried out using 2 M dextrose as the homogenizing solution (results not shown). The results were similar to those found with sucrose, with the exception that less than 2 hours spinning at $100,000 \times$ was required for the nearly complete sedimentation of PNA (due primarily to a lesser viscosity).

Results of PNA distribution studies on egg homogenates prepared in molar glycine. Since glycine has been shown (for references see (11)) to be a major intracellular constituent (concentration estimated at 0.24 M) of the sea urchin egg, homogenates prepared in the presence of this amino acid were investigated. The results of an experiment demonstrating the distribution of PNA and Protein N in separated fractions from an egg homogenate prepared in molar glycine have been summarized in Table IV. Appreciable lysis of echinochrome granules was observed in all homogenates prepared using glycine.

TABLE IV

Distribution of PNA and Protein N in Centrifuged Fractions Obtained from an Egg Homogenate Prepared in Molar Glycine.

Fraction ¹	Centrifugation		Conc./Gm Original Wet Wt. Eggs			
	Force	Time	PNA	Per cent of Total	Protein N	Per cent of Total
Initial homogenate . . .	—	—	8.5 mg	100	16.2 mg	100
Sediment 1	$350 \times g$	30 min.	0.35	4.1	3.4	21
„ 2	$1\,500 \times g$	30	0.44	5.2	2.2	14
„ 3	$25\,000 \times g$	30	0.59	6.9	2.8	17
a) Sediment 4a	$10^5 \times g$	30	3.1	37	1.1	7.0
Supernate 4a			4.1	48	7.0	40
b) Sediment 4b	$10^5 \times g$	120 min.	6.7	79	1.9	12
Supernate 4b			0.27	3.2	6.1	38

¹ Fractions prepared by successive centrifugation of a 1:10 homogenate as designated. Following the $25,000 \times g$ spin, resulting supernate diluted with equal volume of molar glycine and divided equally into 2 tubes (a, b). One tube (a) spun for 30 minutes and other (b) for 120 minutes at $100,000 \times g$. All sedimented fractions treated without further washing for PNA and Protein N analyses.

The results obtained from glycine homogenates are similar to those found with sucrose. Again, less than 20 per cent of the PNA and approximately half of the protein was sedimented at forces up to $25,000 \times g$. A nearly complete sedimentation of PNA was achieved only after 2 hours at $100,000 \times g$.

On the dissociation of PNA from particulate structures of the egg

Distribution of PNA as observed with egg homogenates prepared in isotonic, hyper- and hypotonic KCl solutions. The effect of increasing ionic strength on the relative sedimentability of PNA within centrifuged egg fractions was

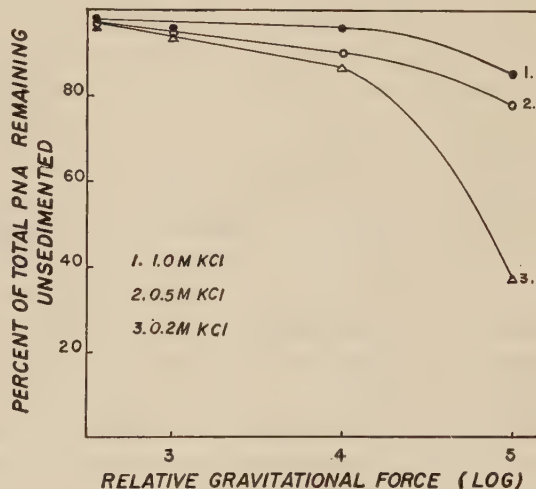


Fig. 1. The effect of KCl concentration on the relative sedimentability of PNA from egg homogenates.

investigated. Homogenates (1:20) prepared in molar, 0.5 M and 0.2 M KCl were subjected to successive centrifugal forces ranging from $350-100,000 \times g$. Centrifugation times were limited to 30 minutes at each of the selected forces. In these experiments, aliquots of the resulting supernates were removed following each successive centrifugation and analyzed for decrease in PNA. In view of the limited quantity of DNA present within the unfertilized egg, no attempt was made to correct for DNA extracted by the stronger salt solutions. Homogenates prepared in KCl showed considerable lysis of echinochrome granules until concentrations of 1 molar were approached where only a minimum of lysis was found. The results of these experiments have been summarized in Fig. 1.

It can be seen from the results presented that with increasing ionic strength

there results a tendency towards a solubilization of the PNA. Similar results have been described by others working with different materials (3, 10). The greater tendency for PNA sedimentation in the presence of lesser concentrations of KCl could not be interpreted on the basis of lesser specific gravity of viscosity (molar KCl and 0.5 M sucrose have nearly identical specific gravities, yet a greater sedimentation of PNA always was found in the sucrose homogenates, which in addition possess an overwhelmingly greater viscosity). A basis for interpreting these results was provided as a consequence of additional experiments which have been summarized in the following section.

The effect of added metal-complexing agents on the distribution of egg PNA. Reports (15) on the electrolyte content of unfertilized *Arbacia* eggs show the presence of a high content of divalent (Ca^{++} , Mg^{++}) relative to monovalent cations (Na^{+} , K^{+}) and a strikingly low concentration of inorganic anions. In view of these findings it is apparent that the anionic constituents of the egg are to a large extent organic in nature and involved in complex bonding with divalent cations. The extent to which PNA (as nucleoprotein?) binding of divalent cations exists and the effect of such binding on its apparent structural association with particulates was investigated.

Egg homogenates were prepared in the presence and absence of added metal-complexing agents. The complexing agents selected for these studies were the sodium salts of citric acid and ethylenediamine-tetraacetic acid ("versene"), both recognized as extremely effective metal-binding agents. The prepared homogenates were centrifuged and the distribution of PNA within the separated fractions was determined.

In Table V are summarized the results of an experiment demonstrating the effect of added "versene" on the distribution of PNA in centrifuged egg fractions prepared in sucrose. A single homogenate was prepared at a 1:10 dilution in 0.4 M sucrose containing 0.1 M trishydroxymethylaminomethane ("tris") buffer at pH 7.4. A suitable aliquot of the homogenate was removed as a control for total PNA and Protein N determinations. The remaining homogenate was divided into 2 equal parts; to one part was added an equal volume of 0.4 M sucrose, to the other was added an equal volume of 0.4 M sucrose containing 2 per cent disodium "versenate". The 2 diluted aliquots (1:20) were then subjected to simultaneous centrifugation and the distribution of PNA and Protein N determined.

From the summarized values it can be seen that the addition of "versene" results in an apparent dissociation of PNA from sedimentable particulate structures. The maximum limit of PNA dissociation by the addition of higher

TABLE V

Distribution of PNA and Protein N in Centrifuged Fractions of Eggs Homogenized in Sucrose (0.4 M) in the Presence and Absence of Added Metal-Complexing Agent (0.027 M "Versenate").

Fractions ¹	Centrifugation		Conc./Gm Original Wet Wt. Eggs			
	Force	Time	PNA	Per cent of Total	Protein N	Per cent of Total
Initial homogenate	—	—	8.0 mg	100	18.2 mg	100
A. Without "Versene"						
Sediment 1	10 ⁴ × g	10 min.	1.3	16	9.1	50
Sediment 2	10 ⁵ × g	60 min.	5.1	64	2.5	14
Remaining supernate . . .			1.8	22	7.2	40
B. With "Versene"						
Sediment 1	10 ⁴ × g	10 min.	1.1	14	8.9	49
Sediment 2	10 ⁵ × g	60 min.	2.8	35	2.0	11
Remaining supernate . . .			4.5	56	7.7	42

concentrations of "versene" was not investigated. Although some solubilization of protein was apparent in the presence of added "versene", the amount was not nearly proportional to that observed with the PNA, suggesting the presence of either chemically heterogeneous particulates or a selective dissociation of chemical groupings from particulates of homogeneous chemical composition. Calculations based on these somewhat crude experiments show a solubilization of 2.37 mg PNA and 2.94 mg Protein (Protein N × 6.25) in the presence of the added "versenate".

The effect on PNA distribution by added metal-complexing agents (citrate in these experiments) was further investigated with respect to egg homogenates prepared in water and hypo- and hypertonic KCl solutions (see Table VI). In the absence of added citrate, the solubilization of PNA in hypertonic KCl (0.7 M) as compared with hypotonic KCl (0.2 M) solutions is again demonstrated. The addition of 0.05 M citrate results in an appreciable solubilization of PNA when used alone or added to the hypotonic KCl homogenates, while having little additional effect in the presence of the homogenate prepared in hypertonic KCl. The inability of citrate to enhance

¹ For details concerning homogenate preparation and centrifugation procedure, see text. All sedimented fractions treated without further washing for PNA and Protein N analyses.

the effect of hypertonic KCl would suggest a common mechanism of PNA solubilization by these separate agents. It is concluded that the mechanism of PNA solubilization involves the displacement of divalent (calcium and/or magnesium) cations, which are intimately associated with either aggregate formation or binding of PNA (presumably as nucleoprotein) onto existing particulate structures. The removal of divalent cations can be accomplished as a result of successful competition by strong complexing agents such as citrate or "versene". On the other hand, a displacement of divalent cations can be expected in the presence of strong KCl solutions as a result of a simple ion exchange reaction.

The results of an experiment demonstrating the relative sedimentability of PNA from an egg homogenate prepared in isotonic artificial sea water have been included in Table VI. A greater proportion of the PNA was found to be sedimented at the lower gravitational force from the sea water homogenate, presumably indicating the presence of a favorable divalent-to-monovalent cation ratio for nucleoprotein aggregate formation or binding.

In the presence of citrate or "versene" a marked stabilization of echinochrome granules was observed as measured by the relative absence of pigment in the supernatant liquor remaining after low speed centrifugation. The ability of citrate to stabilize echinochrome granules has been reported

TABLE VI

Effect of Added Metal-Complexing Agent (Citrate) on the Relative Sedimentability of PNA in Egg Homogenates Prepared in Electrolyte Solutions.

Homog. Media ¹	Per cent of Total PNA remaining non-sedimentable ²	
	After 10 min. at $10^4 \times g$	After 60 min. at $10^5 \times g$
0.05 m citrate	93	60
0.02 m KCl+0.05 m citrate . . .	94	56
0.7 m KCl+0.05 m citrate . . .	88	51
0.7 m KCl	86	46
0.2 m KCl	86	22
Isotonic art. sea water	69	18

¹ All homogenates contained in addition to listed salts, final 0.05 m "tris" buffer at pH 7.4. Citrate used as the trisodium salt. All homogenates prepared at a 1:20 dilution prior to centrifugation.

² Small amounts of DNA solubilized in the hypertonic salt solutions were disregarded. All nucleic acid remaining non-sedimented analyzed as PNA.

previously by others (8, 12). It was found in the present investigations that granule lysis was minimized even in extremely hypotonic solutions (0.2 M KCl or sucrose) if small amounts of citrate or "versene" were added. The *apparent* stabilization by these agents can perhaps be interpreted as the result of a partial destruction of the granule surface by the removal of divalent ions, resulting in an increased porosity and consequent ready osmotic equilibration with the medium.

DISCUSSION

From the observed results it is assumed that the PNA is present within the egg in the form of nucleoprotein aggregates or as nucleoprotein loosely bound to other sedimentable particles. It is further assumed that the binding of nucleoprotein is effected by calcium or magnesium ion (or both) in a loose manner such that the simple displacement of the metal ion from the complex results in a dissociation and consequent solubilization of the nucleoprotein. In view of the prolonged centrifugation at high forces required to effect sedimentation, it is necessary to assume that the PNA is associated with structures of relatively small dimensions (i.e., insofar as well-studied organized cellular particulates are concerned). Although specific experiments were not carried out to determine the relative size of the PNA-associated particles, an approximation may be made from data presented in an earlier section of this report.

Assuming convection-free centrifugation, the following formula has been given by Pickels (16) which is applicable for the estimation of particle size using a fixed angle rotor of the type present in the Spinco Model L Ultra-centrifuge:

$$t = 54 \left(\frac{D-L}{D+L} \right) \left(\frac{\eta}{d^2 (d_p - d_m) N^2} \right)$$

Where t is time (min.), D is radial distance (cm) from outer edge of tube (for complete sedimentation) to the axis, L is the radial distance from the meniscus to the axis of rotation, η is the viscosity of the medium, d is the average diameter of the particles sedimented (cm), d_p and d_m are the densities of the particles and medium respectively and N is the speed in r.p.m. From previously presented data (see Table III) it was shown that an essentially complete sedimentation of PNA occurred only after 3 hours at $100,000 \times g$ in a medium of 0.5 M sucrose. A calculation of particle size based on the time required for complete sedimentation would give one a

value corresponding to the smallest sized particles, assuming the presence of more than one size (i.e., a distribution of sizes). On the basis of such a calculation, the smallest particles containing PNA were estimated to be around 3×10^{-6} cm in diameter, corresponding to an approximate molecular weight of 10,000,000 (assumed spherical). The density of the solvated particles was not determined but assumed for calculation purposes to be near that of typical biological materials (i.e., around 1.2). Further experiments specifically designed to map the distribution of particle sizes should be of interest.

Whether the PNA containing particles present within the *Arbacia* egg represent simple aggregates of pentose nucleoprotein can only be a matter of speculation. That PNA complexes involving metal and protein can and do exist was demonstrated by Henry, Stacey and Teece (9) in elucidating the nature of the Gram positive stain.

The ability of pentose nucleoprotein to form simple aggregates corresponding to the dimensions of the PNA containing particles of the *Arbacia* egg would not appear to be too unreasonable in view of the demonstrated (7) presence of extremely high molecular weights for PNA when prepared under suitable conditions.

SUMMARY

The distribution of PNA within centrifugally separated fractions of the unfertilized egg of *Arbacia* was investigated. The bulk of PNA could be separated within a fraction obtained only after spinning at relatively high forces. An estimation of particle size indicated that the minimum dimension of the PNA associated particulates was around 3×10^{-6} cm in diameter, corresponding to a molecular weight of approximately 10,000,000 assuming a spherical structure. The PNA, presumably as nucleoprotein, was found to be dissociated into a less readily sedimentable form in the presence of strong KCl solutions as well as strong metal-complexing agents such as citrate and ethylene diamine tetra acetate. It was concluded that the dissociation was the result of a displacement of divalent cation (ca ++ or mg ++, or both) involved in either aggregate formation or binding of nucleoprotein to other sedimentable structures.

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QUANTITATIVE β -TRACK AUTORADIOGRAPHY OF SINGLE CELLS

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SEVERAL approaches to quantitative autoradiography have been made along different lines, *viz.* through densitometric evaluation of random grain autoradiographs, through grain counting, and through track counting.

The first mentioned method can be made fairly accurate when special precautions are taken to standardize exposure and processing conditions (3, 7, 12, 13). However, densitometric analyses of the autoradiographs necessitate a separation of the photographic emulsion from the specimen under investigation which, as a rule, involves a sacrifice of resolution. Densitometry provides a measure of the total beta flux reaching the emulsion per unit time and unit area of the film, or specimen, respectively. However, the calculation of the total beta flux from density measurements must be based on calibration curves obtained from beta sources of the same energy and known strength. The minimum area that can be measured by means of a microdensitometer is of the order of 0.1 square millimetre.

The quantitative evaluation of autoradiographs on the basis of grain counting has been undertaken by a group of workers at the Carlsberg Laboratory of Copenhagen (1, 2). They have achieved the determination of the beta flux from a given area of the specimen without sacrifice of resolution. This is due, partly, to the fact that the objects studied are very small. The area under investigation is of a size required for histological studies. The quantitative grain counting as developed by Holter *et al.* is thus superior to the densitometric measurements described in the literature, despite two definite difficulties, *viz.* 1) visual counting of grains under the microscope is extremely laborious and time consuming, 2) the method is applicable to small specimens only or to the study of histological details. The analysis of larger objects, such as ordinary histological sections, will require an enormous expenditure of time and effort.

Quantitative evaluation of autoradiographs on the basis of track counting has been carried out successfully in the case of α -emitters, for example Ra

and Th, deposited in bone or soft tissue (9, 11). α -track counting lends itself most readily to the calculation of the number of disintegrations taking place within a small volume of tissue, especially if the α -emitter is distributed evenly and self-absorption corrections can be applied. In α -track autoradiography a very high resolution can be achieved and the counting is much less strenuous and laborious than is grain counting.

Since α -emitting substances are of very limited interest in biological research, and the majority of tracers applied are β -emitters, the problem arises whether β -particles can be registered as tracks in suitable photographic emulsions and whether β -track counting can be made a quantitative method without sacrifice of resolution.

This method was first applied by Boyd and Levi (4) who obtained β -track autoradiographs from tissue sections containing C-14 labeled glycine. These authors floated the tissue sections on Eastman Kodak NTB-2 plates and they stated that the number of particles per unit time, originating from a given volume of the tissue as determined by β -track counting in the superimposed emulsion, was in fairly good agreement with the result of ionization chamber measurements on pieces of the same tissue.

The method has later been applied by Campbell (5, 6) to distribution studies of Fe-59 in placenta of rabbits. However, Campbell confined himself to relative track counts without comparing the result of track counting with any other method of measuring the amount of tracer present in the tissue. The resolution obtained in these autoradiographs was estimated by Campbell, and by Boyd and Levi (4, 5) to be of the order of 10 μ .

A study of the applicability of β -track counting as a quantitative autoradiographic method and of the resolution that can be obtained has been carried out by the present author and the work is described in detail elsewhere (10). It was found that, with commercial plates and the floating or contact technique, the reproducibility of results is not very good and the efficiency of the plates as well as their resolution poorer than was expected. An attempt has therefore been made to improve the method, at least in the case of soft β -emitters, by applying a liquid emulsion of high sensitivity to medium and low energy β -particles.

In preliminary studies with C-14 standard sources and Ilford G-5 emulsion, it was found that the latter will register low energy β -particles with a much higher efficiency than does the NTB-2 plate. Moreover, resolution is improved considerably by suspending the object to be studied in the emulsion proper. A similar approach has been made previously by King *et al.* (8), using a relatively large cell, *Paramecium caudatum*, labeled with P-32.

METHOD

The cells used in the experiments described below are yeast and algae, the tracers C-14, S-35, and P-32. The labeled cells are washed in inactive nutrient solution of the same composition as that in which they had been grown. Subsequently, a small quantity of the cells suspended in about 50 microlitres of the nutrient solution or of distilled water is added to the molten emulsion.

The G-5 emulsion is available as a gel and comes in small soft pieces of relatively high water content. In the darkroom, a suitable quantity is transferred to a clean test tube or beaker which is then placed in a water bath at about 43° C for 10 minutes. When the emulsion is liquefied, the cell suspension is added with a micropipette and mixing is achieved by gentle rotation and shaking of the glass alternating with immersion of the glass into the water bath to maintain the temperature of 43° C.

"Subbed glass slides" (available from Ilford) are placed on a double metal table provided with an inlet and outlet for circulating water. The table is leveled very carefully and heated to about 40° C by a stream of warm water. When the warm emulsion is poured on the warm glass slides, the emulsion will spread easily to uniform thickness. Subsequently, the warm water is slowly replaced by cold water so that the table is cooled to about 15° C in the course of 2 minutes. On cooling, the emulsion will solidify and set. After another 2-5 minutes, a fan is directed towards the plates and drying occurs in the course of a few hours at room temperature and in the dark. During drying, the metal table is maintained at room temperature; cooling will slow down the drying process, heat might affect the properties of the emulsion. When the plates look dry, usually after 4 hours (inspection under a Wratten 2 safelight at 1 m distance), they are placed in light tight boxes containing a small quantity (c. 10 g) of dry silica gel. Thorough drying is of decisive importance. After drying with a fan, only (30-50 per cent relative humidity of the dark room), the G-5 emulsion will not register low energy β -particles as tracks. On the other hand, it was found that too rigorous drying sometimes causes the emulsion to peel off the glass plates. Therefore, the silica gel is removed from the boxes after 12-15 hours, and exposure is continued in the absence of a drying agent. Processing of the G-5 emulsion is carried out using an amidol developer.¹ It was found that strict temperature development as prescribed for very thick nuclear track emulsion is not required in our case, because the emulsion has never been poured thicker than about 150 microns. However, it is advantageous to presoak the plates for 10 minutes in cold developer (+5° C) and to transfer them to developer at 13° C immersed in a water bath at 28° C, so that a temperature of 20° is reached in the course of a few minutes; actual developing time 18 min at c. 20° C. From the developer, the plates are transferred to distilled water, to a 1 per cent acetic acid stop bath, and then to 30 per cent hypo. Fixing requires 3-5 hours. Finally, the plates are washed in running tap water for at least 2 hours. During processing, the plates are kept in horizontal position, so that distortion is avoided.

¹ 2.2 g sodium sulfite in 100 ml water and 0.46 ml sodium bisulfite (sp. gr. 1.34) in 210 ml water are mixed, subsequently 1 g amidol is added to the mixture. Makes a total of 310 ml which is prepared fresh. This developer cannot be used after storage.

RESULTS

Yeast cells and *Scenedesmus* algae are suitable for these experiments, because they are well preserved morphologically after embedding in a photographic emulsion and subsequent processing of the emulsion in a developer of pH 7.5.

In a thick emulsion, some difficulty sometimes arises with respect to the visibility of the yeast cells. The refraction indices can be such that the cells cannot be seen in a completely dry emulsion. It usually helps to moisten the plate in 70 per cent alcohol prior to inspection. Therefore, mounting of the plate with canada balsam and a cover slip must be omitted. Algae always appear distinctly, their color is preserved, and nuclei can be distinguished. The β -tracks from C-14 and S-35 labeled cells are easily recognized and counted, they have an average length of 10 grains and, mostly, their points of origin seem to lie directly on the cell surface. However, tracks do occur which seem to start at some distance from the cell. Whether this is due to occasional failure of the emulsion to register the beginning of a track, or to slight distortion of the emulsion, is unknown at present. It is of importance that the cells to be studied be sufficiently far apart in the emulsion so that their track patterns do not overlap. Moreover, it is desirable to evaluate the track background in cell-free areas not too far away from the active cells.

When the cells are labeled with P-32, the tracks are much longer and more irregular. Unless the cell is situated in the centre of a very thick emulsion, high energy β -particles originating from the cell will leave the emulsion and undergo back-scattering from the glass slide or the air. They can then re-enter the emulsion at considerable distance from the cell and from another track. We cannot expect, therefore, to see all tracks around a P-32 labeled cell originate from the cell proper. For the same reason, the track count cannot be as reliable a measure for the number of disintegrations which has taken place in the cell as in the case of a soft β -emitter.¹

The autoradiographs of single cells which have been grown in a labeled nutrient solution for a period of a few days (48 hours in the present case) confirm what should be expected in view of the fact that the cell population was not grown under conditions which would produce physiological homo-

¹ King *et al.* (8) in their paper on P-32 labeled *Paramecium* do not mention which fraction of the tracks observed actually starts at the boundary of the cell.

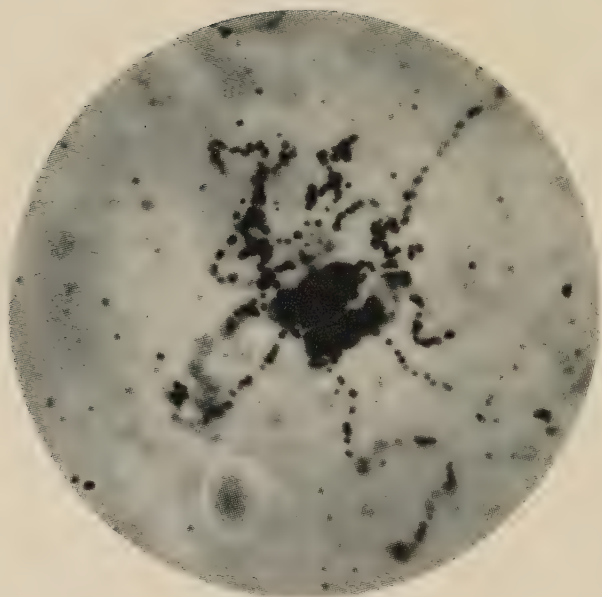


Fig. 1. C-14 labeled *Scenedesmus* algae suspended in Ilford G-5 emulsion. Beta tracks from radiocarbon. Magnification 900 $\times$.

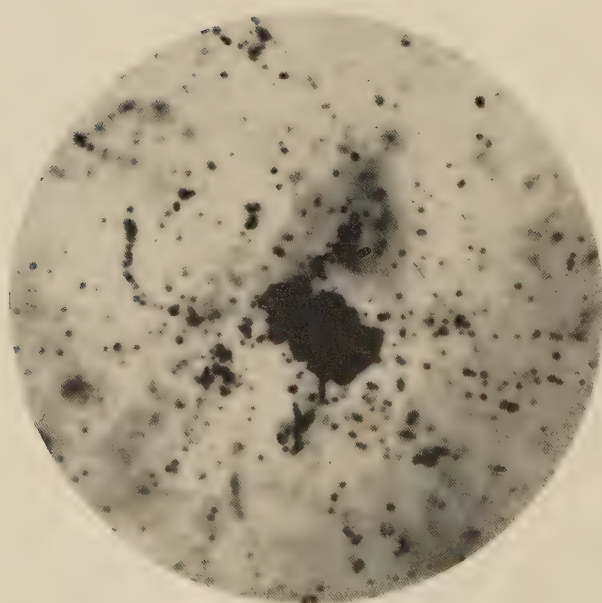


Fig. 2. P-32 labeled *Scenedesmus* algae suspended in Ilford G-5 emulsion. Beta tracks from radio-phosphorus. Magnification 900 $\times$.

geneity, *viz.* that the uptake of the labeled ions varies greatly from cell to cell, and depends on the physiological state of the cell. A suitable tracer level or, correspondingly, a suitable exposure time can thus be estimated only on the basis of the average uptake in a large number of cells. For every exposure time, some cells will appear to be entirely inactive, others will give a track pattern that is suitable for counting, and others, in turn, will be overexposed. A quantitative evaluation of the number of disintegrations registered per cell will therefore include not only the statistics of the decay process, but also the variation in the uptake of the tracer due to different cellular activity. In other words, the method described above, while enabling us to observe the turnover in the individual cell, requires an evaluation based on extensive statistical material.

DISCUSSION

Apart from the advantage of observation of tracks from individual cells, the track counting method requires a still lower level of activity than does the grain counting method. According to different authors, every low energy β -particle reaching a 4 micron autoradiographic stripping emulsion will give rise to 1 or 1.5 grains. It is not easy to detect with certainty a few grains above background around a cell. A small number of tracks emerging from a cell, however, can be detected and counted very easily, and the resolution will be at least as high as, or higher than, in the stripping film autoradiography *ad modum* Pelc. On the other hand, exposure of the autoradiographic stripping film used in grain counting can be extended over periods of weeks without undue increase in background. Even during storage in the cold, the G-5 emulsion remains sensitive to low energy β -particles only for about 2 or 3 weeks, and during exposure, the emulsion will accumulate track background from cosmic radiation which, however, can be suppressed considerably by storing the plates in a lead shield inside the refrigerator.

The question then remains to be answered whether the counting of β -tracks originating from a cell suspended in a nuclear track emulsion is a reliable measure of the number of disintegrations which occurred in the cell. In the first place, it is required that the cell be surrounded by emulsion on all sides to a thickness roughly corresponding to the range of the β -particles in emulsion. Since the density of G-5 is approximately 4, the mean length of the zig-zag track will be of the order of 25 microns. β -particles originating from the cells situated 15–20 microns from the (upper or lower) surface of the emulsion layer will be recognized as tracks. Actually, it is very easy to

see in the microscope whether or not a cell is suitably located within the emulsion layer.

Secondly, it is of importance to ascertain that the labeled ion or compound does remain in the cell during preparation for autoradiography. Leaching of the tracer into the emulsion is frequently observed in any one of the "wet" autoradiographic techniques available, and will invalidate the counting result. The only check on this source of error seems to be a comparison of the number of background tracks in emulsion containing labeled cells with that of a cell-free control. In the case of *Scenedesmus* algae grown in C-14 labeled nutrient solution, and of yeast labeled with S-35, we have not been able to detect an increased track background of the cell containing emulsion over the control.

Finally, reproducibility of the properties of the emulsion is of decisive importance in quantitative work. There is little reason to believe that a properly treated fresh emulsion fails to register electron tracks under controlled conditions. A systematic study of this type could not be found in the literature. The present author has collected data on the homogeneity of nuclear track emulsions manufactured in the U.S.A. These data indicate that inhomogeneity of plates does play a part in quantitative track counting. However, inhomogeneity of plates does not contribute more to the total error involved in track counting than do several other factors inherent in the method (10).

When considering the applicability of the technique described above, the following factors must be kept in mind. As long as we wish to work with emulsion thicknesses which do not require elaborate precautions during processing, soft β -emitters, only, can be used. The tracer must be present in the cells as a compound hardly soluble in water. The size of the objects to be studied is of some importance, too. When dealing with objects which are large as compared with the mean track length, it will be difficult to disentangle the tracks shadowed by the object. In this respect, algae and yeast cells have a suitable size. Even bacteria lend themselves to investigations by means of the technique described.

SUMMARY

A method of high resolution beta track autoradiography applicable to single cells (such as yeast or algae) is described. The cells are suspended in liquid G-5 emulsion of Ilford and low energy beta tracks from C-14 and S-35 are registered quantitatively.

The author wishes to express her appreciation to Dr. V. Hartelius of the Carlsberg Laboratory, Dept. of Physiology, for growing the S-35 and P-32 labeled yeast cells, and to Dr. C. Chapman Andresen of the Carlsberg Laboratory, Dept. of Cytochemistry, for growing the C-14 and P-32 labeled algae. My thanks are also due Dr. H. Holter, Carlsberg Laboratory, for stimulating discussions.

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DIFFERENTIATED MAMMARY GLAND TUMOR TISSUE GROWN IN AN ALKALINE MEDIUM *IN VITRO*¹

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IN the course of our work on the mammary tumor agent, several strains of mammary tumor cultures were maintained in Carrel flasks for various purposes.

As repeatedly described by different workers, such cultures, depending on the stroma content in the explant, grow either as undifferentiated sheets of squamous epithelium, or as mixed cultures, where epithelium and fibroblasts grow for long periods together.

Undeveloped acini have sometimes been seen around the explants, but they usually disappeared after one or two transplantations.

In cultures which were always grown in a medium containing chicken plasma, embryonic extract, horse serum and Earl's saline of pH 7.4, a saline of pH 9.3 was used, for some time, by error. Strains of mouse fibroblasts and tumor strains in which the fibroblasts dominated, degenerated and had to be discarded, whereas the cultures which contained rudiments of mammary gland tissue developed luxuriantly. The fibroblasts in these cultures disappeared completely, but ducts and lobules of acini, growing richly in all directions around the explant, presented a most unusual picture (Fig. 1). The well developed acini had enlarged end-buds, where a rather thick wall, lined with two or more layers of cuboidal cells, encircled a large lumen filled with a coarsely granulated material (probably secretion) (Fig. 2). Sudan-Haematoxilin staining revealed fat granules scattered in the lumen, but not all the contents consisted of fat, since basophile blue granules were seen among the red ones.

Such differentiated cultures are now being maintained, using Earl's saline adjusted to pH 9-9.3. They are fed with Earl's mixture twice weekly and transplanted every 3-4 weeks. For transplantation each culture is cut into 2-4 fragments. The fragments growing near the bottom of the flask grow slowly, but develop into differentiated glandular tissue only, whereas in the fragments growing nearer the surface of the plasma, rapidly growing epithel-

¹ Aided by a grant of the Damon Runyon Memorial Foundation.

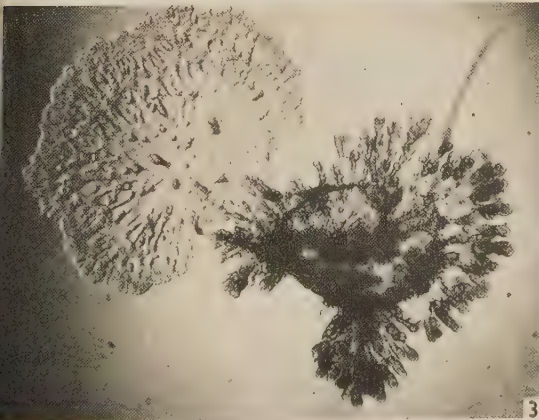
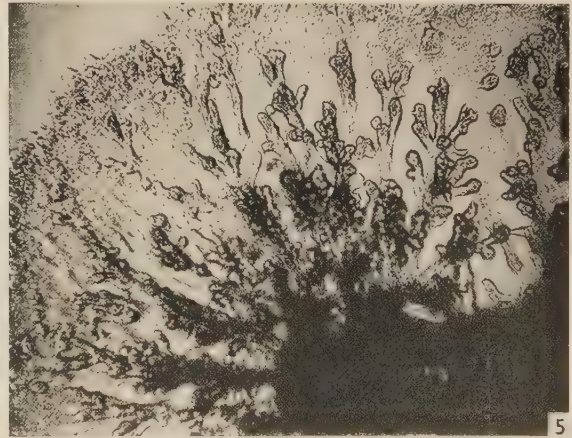
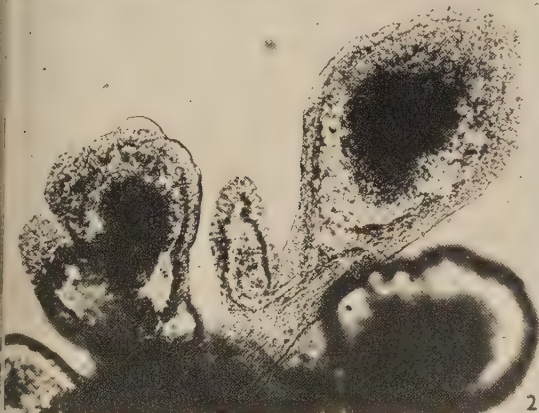
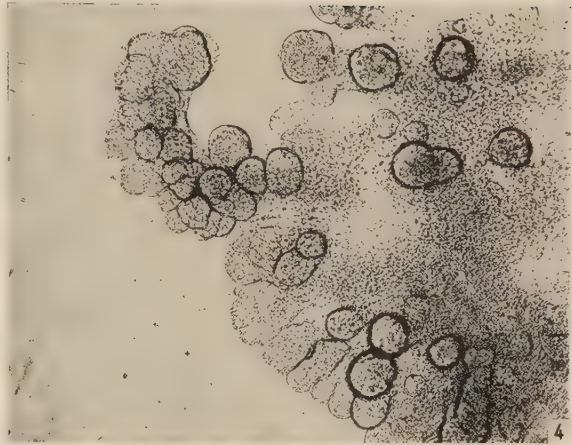
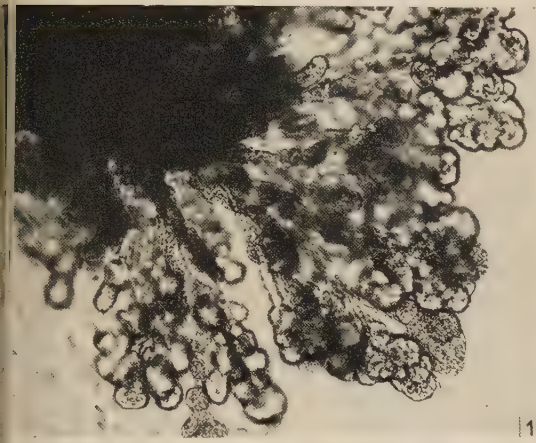


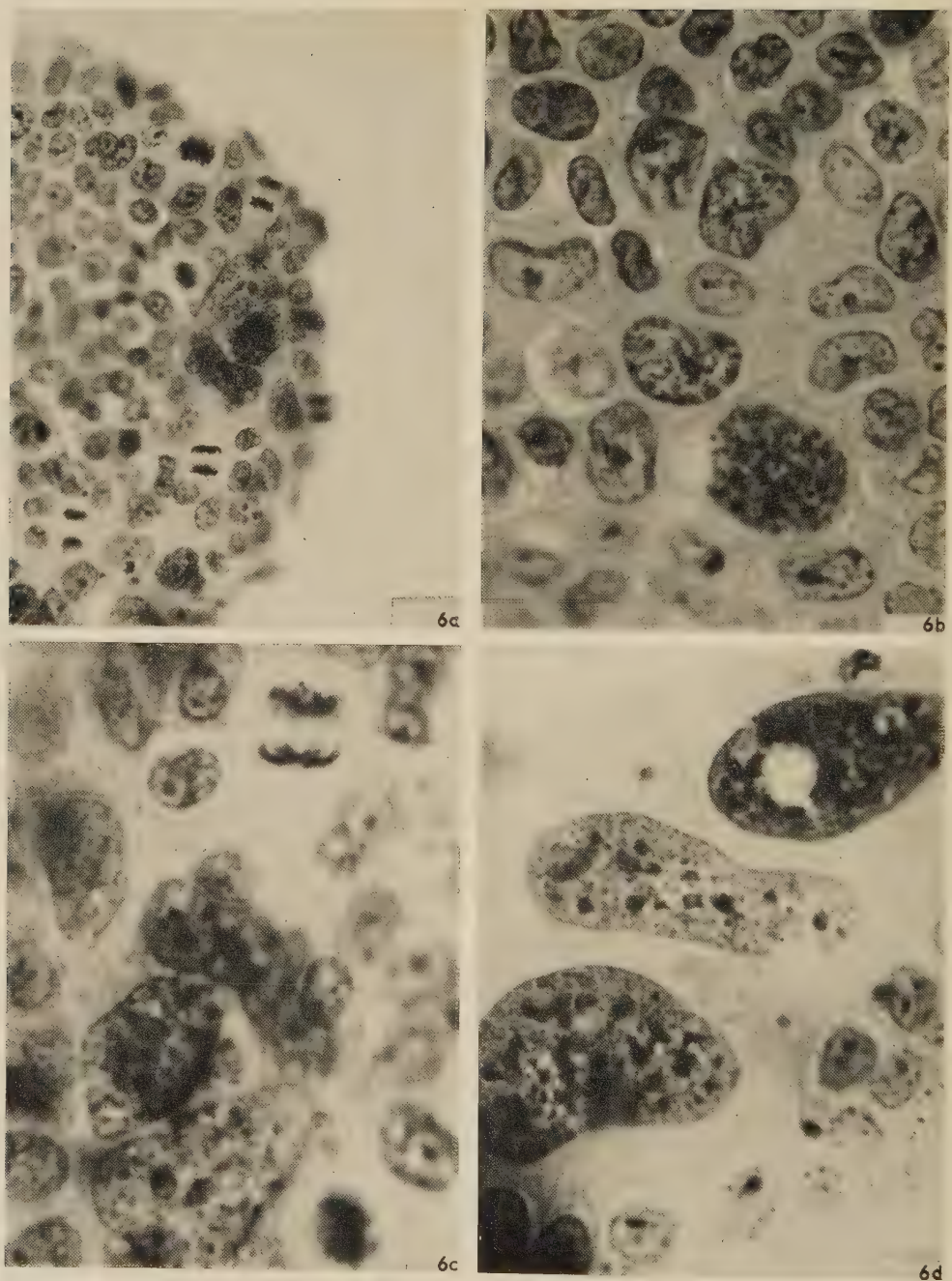
Fig. 1. Part of a culture of series 708, showing lobules of acini with end buds. Cultivated 500 days. Photographed 11 days after the 22nd transplantation. $\times 110$.

Fig. 2. End buds at high magnification. Note thick wall and granulated content. $\times 160$.

Fig. 3. Whole culture of series 826, showing part of culture growing as ducts and acini and part as an organised epithelial sheet grown at the site of the incision. Cultivated 134 days. Photographed 19 days after the 5th transplantation. $\times 51$.

Fig. 4. Part of a culture of series 708, showing at high magnification a dedifferentiated sheet, beginning to organise on the periphery. Cultivated 270 days, 11th transplantation.

Fig. 5. Part of a culture of series 708 showing an epithelial sheet where acini formed around the explant. Cultivated 442 days, 17th transplantation. $\times 120$.



ial sheets appear at the site of the incision (Fig. 3). Such rather thick sheets which are dedifferentiated at first, gradually begin to organize. Acini usually form first at the periphery (Fig. 4) and later around the explant (Fig. 5). Many mitotic figures appear in the sheets during the organization of acini (Fig. 6 a). There is marked polymorphism of cells and nuclei (Fig. 6 b), giant, polyploid nuclei (Fig. 6 c and 6 d) are often encountered in the sheets, but not in the cells, forming the walls of the acini.

Our first strain (series 708) which was accidentally obtained from a spontaneous RIII tumor, is now 20 months old and is at its 27th transplant generation, preserving all the earlier characteristics.

It should be noted that after 17 transplantations at pH 9.3, some cultures of the series 708 were divided and in addition to the usual medium of pH 9.3, also planted at pH 7.4–7.8. The acini developed normally, but the cultures degenerated earlier than those grown at pH 9.3.

The above results obtained through an error in technique, were subsequently produced intentionally.

Using the alkaline medium and parallel controls at pH 7.4 in growing several samples of adenocarcinoma of the RIII strain, we again observed that acini disappeared in all control cultures after one or two transplantations, whereas in some, though not in all, the cultures grown at pH 9.3, the acini developed richly and were easily transplantable, resulting in 3 strains morphologically identical with the original strain 708. One of the new strains (series 826) is now 11 $\frac{1}{2}$ months old and is at its 16th transplant generation.

DISCUSSION

Growth of carcinomatous tissue at such high pH was, apparently, not recorded before. Fisher (7) noted, however, that pH 7.8 was favorable rather than damaging to mouse carcinoma cells, whereas for fibroblasts the optimal conditions were at pH 7.4–7.8. The damaging effect of alkaline media on fibroblasts was also stated in another publication (8) and later confirmed by other workers.

The ability of carcinomatous tissue to grow luxuriantly at pH 9.3, damaging for connective tissue elements, was evidently an easy method for obtaining

Fig. 6a, b, c and d. Stained sections of parts of cultures from series 708. Harris Haematoxylin.
Fig. 6a. Note numerous mitoses and cell polymorphism. $\times 560$.
Fig. 6b. A part of an epithelial sheet showing cells of various shapes and sizes. $\times 1500$.
Fig. 6c. Cells with giant nuclei. $\times 1500$.
Fig. 6d. Exceptionally large polyploid nuclei. $\times 1500$.

cultures without any admixture of fibroblasts, but it was rather unexpected, that it would lead to the development of such highly differentiated glandular tissue, since Drew (4), Ebeling and Fisher (5) as well as Chlopin (3) advocated the admixture of fibroblasts as a means to obtain acini formation in epithelium of mouse mammary tumor.

Cameron (1), on the other hand, observed formation of acini in cultures of epithelium from human mammary carcinoma, when the cultures contained only a few or better no fibroblasts at all. Later Fisher (9) and Fisher and Parker (10) showed that differentiation is not necessarily dependent on interaction of different tissues, since tubulae appear in cultures of mammary tumor if they are "badly nourished and poorly aerated". Parker (11, 12), using his method of prolonged cultivation without transplantation, was apparently able to obtain cultures containing poorly developed acini. More modern work showed, that the inhibition of proliferation is not the only method of obtaining tissue differentiation, if suitable methods, media (6) and t^0 of incubation (2) are used.

Our observations show that the use of a highly alkaline medium proves to be a good method to obtain from mammary tumors pure cultures of mammary glands with well developed ducts, lobules of acini and typical endbuds without any admixture of connective tissue elements.

Inoculations of cultures into mice. The malignancy of the two morphologically identical series 708 and 826 was tested by inoculations into female and male mice of the RIII strain.

For this purpose, one or two cultures belonging to the same serial passage were inserted into a canule, mounted on a tuberculine syringe containing 0.2 cc of Ringer solution and injected either intramuscularly into the thigh, or subcutaneously into the inguinal region.

The cultures of series 826 belonging to serial passages 2, 3, 5, 6, 8, 11, 14 and 15th were all inoculated into 12 direct descendants of the donor of the tumor taken for cultivation. Rapidly growing tumors appeared at the site of inoculation after an average period of 17 days in all the inoculated mice.

In series 708, the cultures of the first and second passage were inoculated into 2 daughters of the donor, a third daughter and her brother mate being left for breeding. Unfortunately they died without progeny, thus ending the subline of the donor, so that the cultures of the subsequent 20 passages were inoculated into 27 mice belonging to another subline of the RIII mice. The first and second inoculation resulted in tumors at the site of inoculation, but no tumors appeared in any of the other 27 mice.

It is evident that the striking difference in the results of inoculation of the

two morphologically identical series of cultures was probably not due to a difference in their malignancy, but it is more likely that a genetic factor was involved.

Since it is presumed, that gene mutation occurring in an inbred strain of mice may produce a subline, where the transplantability of the tumor of the strain is affected, it is also possible that the lack of takes of all cultures of series 708, except those inoculated into direct descendants of the donor is due to a gene mutation, which may have occurred either in the subline of the donor, or in the subline of the recipients of the cultures.

On the other hand, the inoculation of cultures of series 826 into mice belonging to the subline of the donor demonstrated, that highly differentiated cultures of mammary glands, deriving from a mammary tumor and grown for 11 months (15 passages) *in vitro*, preserved their malignancy, since they all produced rapidly growing tumors at the site of inoculation. These tumors were all adenocarcinomas of the same type as the tumor taken for cultivation.

The presence of the Milk Agent in these cultures is being investigated and will be discussed in another publication.

SUMMARY

A method is described whereby mouse mammary tumors of the RIII strain were grown for long periods in a highly alkaline media.

Connective tissue elements disappeared completely, while a highly differentiated, richly growing glandular tissue developed and could be maintained for indefinite periods *in vitro*.

The inoculations of cultures after various periods of cultivation into RIII mice belonging to the subline of the donor, all resulted in rapidly growing tumors at the site of inoculation.

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A QUANTITATIVE STUDY OF AUTOGENIC AND ALLOGENIC TRANSFORMATIONS IN PNEUMOCOCCUS

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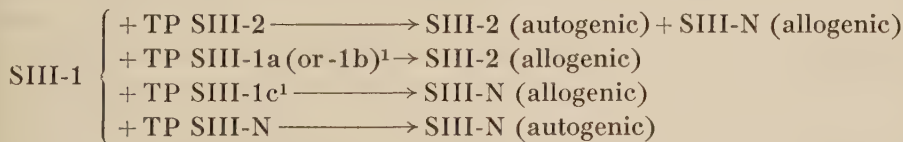
ONE of the major difficulties in the study of capsular transformations in pneumococci has been the lack of a quantitative method for measuring the incidence of transformations in populations treated with transforming agent. The frequency of transformation under optimal conditions (roughly about 0.1 to 0.5 per cent) is so low as to render impractical any attempt to determine quantitatively at the cellular level either the kinetics of transformation or the role of various environmental factors so long as there are no means of selectively eliminating the untransformed cells. A quantitative method has become especially desirable for capsular transformations, however, because of the recent discovery of allogenic transformations (3). The fundamental similarity of allogenic transformations to genetic recombination in viruses and to crossing-over between gene complexes in higher organisms immediately suggests that studies of their relative frequency would be of great interest.

In order to develop a quantitative method for measuring the frequency of capsular transformations, advantage has been taken of a previous observation (7) that pneumococcal colonies often contain transformed cells when grown in a semi-solid agar medium containing a transforming agent. It seemed possible that the frequency of *colonies containing transformed cells* could be measured with reasonable ease and accuracy even if the numbers of transformed cells per colony could not, and that the study of such frequencies might indirectly provide a means of determining the kinetics of transformation. The object of the present investigation was to determine whether the technique of transforming colonies in semi-solid medium can be adapted for purposes of assay. A serious handicap turned out to be the difficulty in reproducing exactly the conditions for transformation, doubtless owing to the complexity of the culture medium employed. Despite this fact, however, certain quantitative relations were consistently observed. For example, evidence of a linear relation between frequency of transformation

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and concentration of transforming agent was obtained in experiment after experiment. Moreover, the method has proven highly sensitive in measuring the action of controllable environmental factors on transformation. The usefulness of this method in quantitative studies therefore seems assured.

Because of the interest that allogenic transformations afford, most of the present studies have been concerned with two mutated forms of the transforming agent affecting synthesis of Type III capsular polysaccharide. These two agents are found respectively in mutant strains SIII-1 and SIII-2; cells of the former strain produce only traces of Type III polysaccharide while cells of the latter synthesize considerable quantities which are, nevertheless, less than those found in the normal smooth (SIII-N) strain. When SIII-1 cells are treated with the transforming agent (TP) extracted from another mutant strain, cells are often produced which are unlike those of either the treated strain or of the strain furnishing the transforming agent. Such transformations are called *allogenic* in contradistinction to the classical *autogenic* transformations, in which the characteristic property of the TP-donor strain is conferred on a cell. The various capsular transformations that SIII-1 cells are known to undergo under the influence of different TP's are the following:



It will be seen that the action of TP SIII-2 upon SIII-1 cells results in both autogenic transformations (SIII-2 cells) and allogenic transformations (SIII-N cells). This pair of transformations has been analyzed in particular by means of the quantitative method described in this paper, and new information has been obtained which limits to a certain degree the possible hypotheses which can be formulated to explain allogenic transformations.

A preliminary report of the findings described in this paper has previously been made (8).

MATERIAL AND METHODS

Theory of the method. Essentially, the present quantitative method consists of inoculating several pneumococci into a deep dish containing a suitable

¹ Strains SIII-1, -1a, -1b and -1c have been derived independently and, although indistinguishable morphologically and serologically, possess different agents having the transforming properties indicated above.

medium for transformation and a desired amount of transforming agent. The entire medium is rendered semi-solid by the dispersion of a small amount of melted agar in the medium. The inoculated cells multiply and give rise to colonies loosely held in place by the gel. After an appropriate period of incubation, the visible colonies are removed one by one and individually put into homogeneous suspension; a sample of each suspension is streaked on the surface of a solid medium that permits one to recognize any transformed bacteria that may be contained in the sample population. Assuming that transformations cease to occur some time before the colonies are isolated, and that the samples plated are sufficiently large, this technique can be used to measure the number of colonies in which one or more transformations have occurred during the course of growth in the semi-solid medium.

The assumption that transformations cease to occur long before the time of removal of the visible colonies (at about 17 hours of incubation) is based on the finding of McCarty, Taylor and Avery (7) that there is a limit to the time in which pneumococci remain capable of reacting with TP. When one inoculates 10^4 cells into 2 ml of the currently employed transformation medium, it takes about 2 hours for the cells to become ready ("sensitized") to transform, after which there is a period of only one hour during which transformations can occur (5). This fact can be easily demonstrated in liquid cultures by adding TP at different times after inoculation. However, the loose gel of the semi-solid medium makes it technically impossible to mix TP homogeneously after the agar sets and the exact duration of transformability cannot be determined. It may be expected, however, that a limited period of transformability prevails in semi-solid cultures as it does in liquid cultures, and that the effective time of exposure of the bacteria to TP is limited by the properties of the system as a whole.

It should be pointed out, however, that the practice of studying only a *limited sample* of the colony formed in semi-solid medium assures in itself that only transformations occurring within a controlled period of time are scored. In the present method a sample of about 10^5 cells from a colony containing over 10^7 cells are examined as a standard procedure. This means that whenever the proportion of transformed cells per colony is 10^{-5} or less, the likelihood of detecting the transformed cells is exceedingly small. Consequently, the method is unlikely to detect cells transformed after the number of cells in the colony exceeds 10^5 . If, however, there is strong selection for or against the transformed cells during growth of the colony, the time within which transformations are detectable is increased or decreased as

the case may be. Assuming that the selection pressure is not a function of the time of occurrence of the first transformation (i.e., the initial proportion of transformed cells), the time in which transformations are detected remains constant in these experiments.

Another factor which obviously must be controlled in a kinetic study of a reaction is the concentration of the reactants. In pneumococcal transformation the reactants are presumably units of transforming agent and the bacteria or some primary cell-receptors. It must be understood, however, that in a semi-solid medium the reactants are not the bacteria originally inoculated, but rather the clones (colonies) to which they give rise. Furthermore, the number of bacteria per colony increases with time during the period in which transformations take place. In order to consider the clones exposed to TP as equally transformable targets, it is necessary that the size of the colonies be uniform and that the number of transformable cells be the same in all colonies. The former requirement seems fulfilled on the grounds that the sizes of colonies, as determined by surface platings of diluted suspensions, do not differ significantly either when the colonies are grown in the same medium or in media differing by a tenfold concentration of TP (Table 1). The latter requirement is supported in those cases reported below where (a) a continuous linear increase in the frequency of colonies containing transformations follows upon increasing the concentration of TP and where (b) all of the colonies contain transformations at sufficiently high concentrations of TP.

In those experiments where the influence of the concentration of TP is examined, the number of clones per volume of medium is effectively fixed by maintaining a standard inoculum size per standard volume of semi-solid medium.

Strain employed. The SIII-1 strain employed in all these experiments is derived from a rough cell of strain R36A transformed in 1950 by the action of the TP extracted from mutant strain SIII-1. The strain was maintained by frequent transfer in medium 3 (3) containing 0.1 cc of rabbit blood per 5 cc of medium.

Preparation and standardization of TP. All TPs studied were prepared according to the method described by Ephrussi-Taylor (3). The TPs were dissolved in physiological saline (0.85 per cent NaCl) solution and kept over chloroform in a sterile tube. Fresh dilutions were prepared from the stock TP solution for each new experiment.

The TP SIII-2 employed was prepared from an SIII-2 strain that had been produced by the transformation of a rough bacterium (strain R36A) by the SIII-2 agent. The TP SIII-N was prepared from an SIII-N strain which was obtained by

transformation of an SIII-1 cell by the transforming agent of normal smooth strain A66. The TP SIII-1c was prepared from a strain produced by the transformation of a rough (R36A) cell by the agent extracted from mutant strain SIII-1c.

The stock solutions of TP were standardized on the basis of their desoxyribose content (2).

Technique of transformations in semi-solid medium. The following standard procedure was adopted for experiments in semi-solid medium. A flask of Medium 3 (pH 7.6 to 7.8) containing 0.2 per cent serum albumin, M/3000 sodium pyrophosphate and a given concentration of TP SIII-2 is warmed to 45° for five minutes in a thermostatically-regulated water-bath. The amount of the mixture employed is 25 cc for each dish of semi-solid medium desired. An appropriate amount of melted 4 per cent sterile Difco-agar is then added to the medium by means of a pipette previously warmed by having been passed several times through a flame; the pipette is drained of its contents and rinsed several times with the liquid contents of the flask. Unless otherwise stated, the amount of agar delivered is that required to produce a final concentration of 0.3 per cent. It will be recalled that specific antibodies are not essential for transformation if the medium is rendered semi-solid by the addition of agar (7).

A small sample of an appropriately diluted culture of SIII-1 cells is then inoculated into this mixture. The contents are rapidly mixed and poured into sterile Pyrex weighing-dishes (5 cm diameter, 3 cm height) having ground-glass lids. In order to prevent contamination, weighing dishes with lids fitting around the upper, outer edge have been chosen. The culture dilution and inoculum size are such as to provide about 50 colonies per dish. The dishes are incubated at 37° for 17 to 20 hours.

The SIII-1 cultures used for inoculating the semi-solid media are grown out in 5 cc of Medium 3 from transfers of 0.05 to 0.1 cc of the refrigerated culture prepared in the preceding experiment. Inocula are never grown out from cultures refrigerated more than two days; if longer intervals elapse between experiments, the refrigerated culture is transferred once or twice before use. The 5 cc inoculum cultures reach maximal optical density in about 4 hours' time, when started from 0.05 cc of the preceding day's culture. Preliminary experiments suggest that an extra half-hour's incubation enhances transformability of the pneumococci, and therefore this additional period of incubation has been made part of the standard procedure. Dilutions are made in Medium 3, which is chilled if more than 15 minutes are required between the preparation of the dilutions and the inoculation of the semi-solid media. Microscopic examination of Gram-stained samples of cultures used in these experiments has shown that about 60 per cent of the bacteria are typical diplococci, that 20 per cent of the bacteria are in four-celled chains, and that only about 20 per cent are in chains of six or more cells. The majority of the colonies formed in semi-solid media, therefore, are derived from diplococci.

Detection of transformed cells in colonies. The colonies that form in semi-solid medium are quite characteristic of the strain employed, provided that the agar concentration is not too high. At agar concentrations of about 0.2 per cent, SIII-1 colonies are distinguishable from SIII-2 and SIII-N colonies in being much denser and apparently firmer. The smoother the type of cell in the colony, the "puffier" the colony. Consequently, SIII-1 colonies in which transformations have occurred very often have a special shape. As was previously observed for rough (R) colonies (7),

the ordinarily spindle-shaped colonies often contain a single "puff-ball" sector containing transformed cells. The resulting appearance is that of an SIII-1 colony with a "pompon" issuing from the side. However, the observation of "pompons" is not an adequate criterion for the occurrence of transformations, for transformations may occur without causing any noticeable prominences, especially at low TP concentrations. These transformations are discovered, however, when the colonies are suspended and plated in the following manner.

The colonies formed in semi-solid media are individually isolated by means of sterile pipettes. Each colony is suspended in 0.10 to 0.15 cc of 2 per cent Difco-Neopeptone and dissociated by vigorous shaking. Colonies contain 1.5×10^7 pneumococci after 17 hours' incubation. About 2×10^5 of the pneumococci from each suspension are streaked out on the surface of a blood agar medium (Medium 2; see 3) which permits the morphological differentiation of colonies derived from transformed (SIII-2 or SIII-N) and untransformed (SIII-1) cells.

The plates are incubated at 37° for 17 hours and examined with the aid of a binocular microscope at a magnification of 20×. The colonies isolated from the semi-solid medium are scored as containing no transformations if no SIII-2 or SIII-N colonies are found in the streaks; they are scored as positive (i.e. clones in which at least one transformation had occurred) if one or more SIII-2 or SIII-N colonies are found.

When widely separated on a surface, colonies of SIII-1, SIII-2 and SIII-N pneumococci are easily recognizable by the degree of their smoothness and by their size. SIII-1 colonies are definitely rough. SIII-2 colonies are smooth, but lack the mucoid, "oil-drop" appearance of SIII-N colonies. The average diameters of SIII-2 and SIII-N colonies after 17 hours' incubation at 37° on Medium 2 are respectively two and three times those of an SIII-1 colony (4). Even when growing closely packed among SIII-1 colonies, the smoother colonies are readily detected, although it is not always possible to determine whether they are composed of SIII-2 or SIII-N cells. When identification is doubtful, cells from colonies are transferred to fresh blood agar medium and their identity established.

At sufficiently high concentrations of TP every colony contains some transformed cells (see Fig. 1, 3 and 5). Although no effort was made to count the number of transformed cells per colony, it was evident from an examination of the streaked sample that these cells never constitute the major fraction of the colony. The highest proportion of transformed to untransformed cells ever observed was one to seven. At a given concentration of TP, the proportion of transformed cells varies considerably from colony to colony, a fact which suggests that in different colonies the first transformations occur at different times. Occasionally as few as one or two transformed cells are found in a sample of 10^5 cells; however, such cases constitute an important proportion (20–30 per cent) of the colonies containing transformations only at very low concentrations of TP. These cases suggest, nevertheless, that it may be incorrect to assume that transformations cease long before isolation of the colonies.

When the standard conditions described above are carefully controlled, reproducible results are generally obtained with a given TP. Variations due to changes in the transformation medium will be discussed below.

EXPERIMENTAL RESULTS

*Factors influencing the frequency of transformation**Concentration of TP*

TP SIII-2. The influence of TP concentration upon the frequency of colonies in which autogenic transformations are induced is illustrated in Figs. 1 and 3. In Fig. 1 are presented the results of experiments in which the action

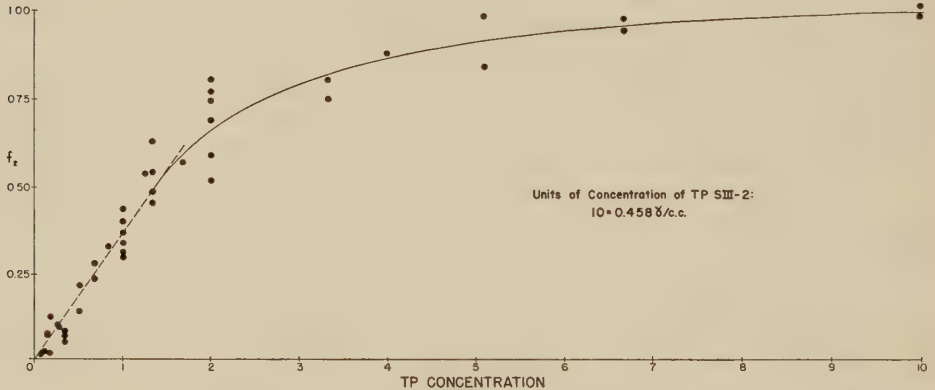


Fig. 1. Frequency of SIII-1 colonies containing autogenic transformations (f_2) as a function of concentration of TP SIII-2. The linear (dashed) portion of the curve was calculated by the method of linear regression ($P \leq 0.01$). Each point represents a measurement obtained in a single experiment in which several concentrations were usually tested (see Table V).

of TP SIII-2 on SIII-1 cells was studied. It should be recalled that this transforming agent induces both autogenic transformations of SIII-1 to SIII-2 and allogenic transformations to SIII-N. In a given experiment the frequencies of colonies containing autogenically and allogenicly transformed cells were determined at the same time. For the sake of convenience, however, the results with autogenic transformations will be discussed at this point separately from those on allogenic transformations. The justification for this procedure will appear below, where the independence of occurrence of the two kinds of transformation will be demonstrated. The data on which Fig. 1 is based are recorded in Table V.

Over the lower range of concentrations of TP SIII-2 the frequency of colonies containing autogenic transformations increases linearly. The linear portion of the curve has been drawn through the data by means of the method of linear regression. It should also be remarked that the linear portion of

the curve passes through the origin of the graph. This finding has two obvious implications. First, it shows that transformations do not occur in the absence of TP. In fact, no SIII-2 or SIII-N cells have ever been observed in SIII-1 colonies which were not treated with TP SIII-2. Secondly, there appears to be no threshold concentration of TP below which the present method is incapable of detecting transformations. This is important, for it means that no transformations are being overlooked either because they occur late or because of strong selection against the transformed cells.

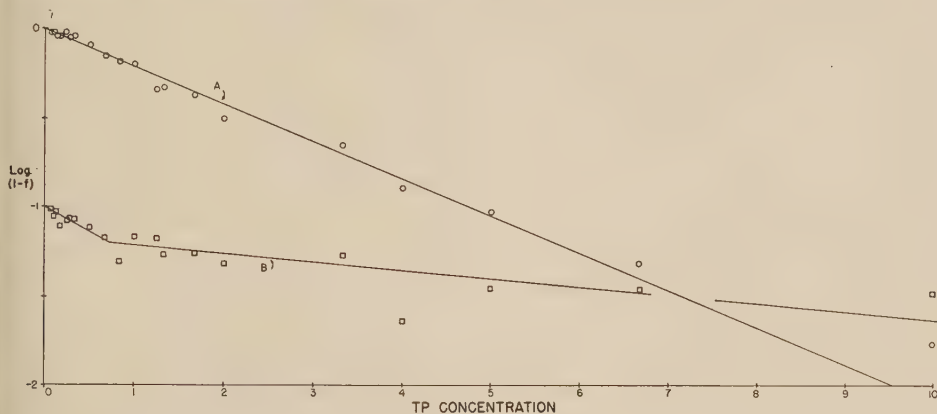


Fig. 2. Logarithm of frequency of SIII-1 colonies containing no transformed cells ($1-f$) as a function of concentration of TP SIII-2. A: autogenic transformations; B: allogenic transformations. In cases where several experiments were performed testing a given concentration of TP, the averages of the measured frequencies are shown. The straight line shown for "A" was calculated by the method of linear regression ($P \leq 0.01$). Curve "B" is displaced one log cycle below "A" in order to make comparison easier.

The linear relation between the frequency (f_2) of colonies containing autogenic transformations and TP concentration no longer holds when over half of the colonies contain such transformations. At concentrations of TP greater than $0.06 \gamma/\text{cc}$ the rate of increase of f_2 gradually slows down, but eventually all the colonies contain some autogenically transformed cells. In order to explain this change in rate of increase of f_2 , it may be supposed that while the number of individual cellular transformations continues to increase linearly with TP concentration, the increase in the number of colonies in which these transformations occur diminishes due to the increased probability for multiple transformations to occur in a single colony at high TP concentrations. Evidence for the increased frequency of multiple transformations at high TP concentration will be given below (see Table V). In other words, each increment of TP concentration may transform the same pro-

portion of the number of organisms which have remained untransformed until then, even though the numbers of colonies "transformed" by successive increments of TP concentration are not equal. If this were so, one would expect the number of "untransformed" colonies to fall off exponentially; that is to say, the logarithm of the number of untransformed colonies should decrease linearly with TP concentration. Fig. 2 A gives such a plot of the data already presented in Fig. 1. It will be observed that the relationship predicted by the hypothesis of strict proportionality between TP concentra-

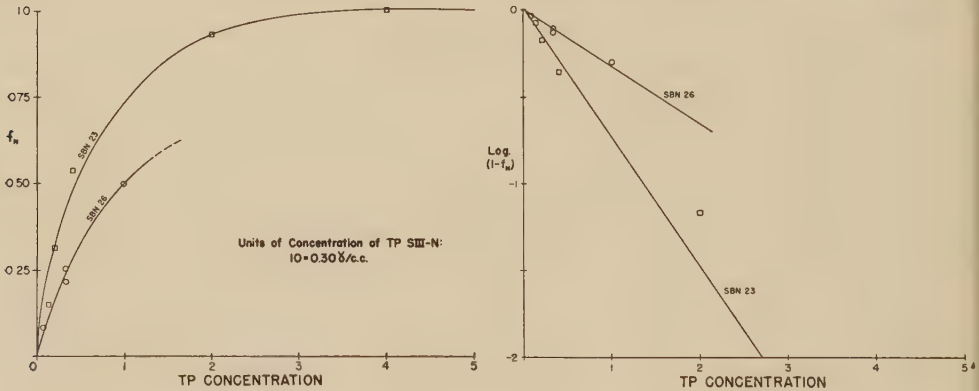


Fig. 3. Frequency of SIII-1 colonies containing autogenic transformations (f_N) as a function of concentration of TP SIII-N. "SBN No. 23" and "SBN No. 26" refer to two different preparations of Medium 3.

Fig. 4. Logarithm of frequency of SIII-1 colonies containing no autogenically transformed cells ($1-f_N$) as a function of concentration of TP SIII-N. "SBN No. 23" and "SBN No. 26" refer, as in Fig. 3, to two different preparations of Medium 3.

tion and transformation is fulfilled. (Analysis of variance shows $P > 0.2$ for equal or greater deviations from linear regression on a chance basis.)

TP SIII-N. In Figures 3 and 4 it may be seen that similar results were obtained with TP SIII-N on strain SIII-1. This transforming agent induces uniquely autogenic transformations to SIII-N. It may be seen that an exponential relation holds between concentration of TP and the frequency of colonies containing SIII-N cells which, as has just been pointed out, is in agreement with the hypothesis of direct proportionality between concentration of TP and frequency of transformations.

Environmental factors

Variations in the medium. The frequency of transformations in SIII-1 colonies is affected by variables other than concentration of TP. Despite sys-

tematic attempts to standardize the preparation of the various ingredients of the transformation medium (albumin, yeast extract, beef serum extract, etc.) it has not been possible to reproduce accurately the conditions for transformation. For reasons as yet not understood, certain preparations of complete Medium 3 were very poor for transformations. With certain preparations it was observed that over a wide range of TP concentrations the frequency of colonies containing transformed cells was constant; for example, with one batch of medium the frequency of colonies containing autogenically transformed cells remained at about 0.10 despite changes in concentration of TP SIII-2 from 0.092 to 0.23 γ /cc (compare with frequencies given in Fig. 1). For this reason, the data presented in Figs. 1 and 5 have been selected from experiments employing either a single batch of Medium 3 or batches giving similar results.

Variations in the rate of increase of the frequency of colonies containing transformed cells, due apparently solely to differences between successive batches of transformation medium, are illustrated in Fig. 3. Apparently certain factors in the medium limit the rate of transformation and differ in amount in different preparations of medium. It is of obvious importance to determine the nature of these unknown factors.

Agar concentration. Even with the same batch of medium a certain degree of variability may be observed between successive TP titration experiments. Variations in agar concentration were investigated as a possible cause of this variability. Several experiments were performed in which the agar concentration was varied between two extremes, one being just high enough to assure firmness of the medium and cohesion of the colonies (0.15 per cent), the other being the standard concentration (0.3 per cent) employed in the titration of TP. The concentration of TP was kept constant in these experiments. At concentrations of agar above 0.3 per cent the colonies become extremely dense and very difficult to disaggregate into homogeneous suspensions. At the two extremes of agar concentration there is a great difference in size (length and diameter) of the colonies, which is due solely to a difference in cell density. When equivalent samples of colonies are removed from 0.15 per cent and 0.3 per cent agar and plated, the numbers of cells per sample are found to be the same (Table I). Because of the extreme looseness of the medium containing 0.15 per cent agar, samples of medium taken between colonies were examined to check that no contamination was taking place between colonies. These controls were all negative in the experiments to be discussed here.

Table II presents the results of two experiments in which a concentration

TABLE I

A. Number of Cells in Colonies Grown in Medium 3 Containing Either 0.12 or 0.012 γ /cc of TP SIII-N.

Colony No.	0.12 γ /cc	0.012 γ /cc
a	39.5 ± 1.5	39.0 ± 7.0
b	23.0 ± 3.0	31.0 ± 1.0
c	30.5 ± 3.5	26.0 ± 9.0
d	25.5 ± 4.5	21.5 ± 1.5
	29.6 ± 6.3	29.4 ± 6.5

B. Number of Cells in Colonies Grown in Medium 3 Containing Either 0.15 per cent or 0.3 per cent Agar.

Colony No.	0.15 per cent Agar	0.3 per cent Agar
a	78.3 ± 4.1	78.0 ± 27.8
b	78.3 ± 12.8	60.5 ± 15.8

Colonies isolated after 15 (Part B) or 17 (Part A) hours incubation at 37° , suspended in 1.0 ml 2 per cent Neopeptone, diluted to 10^{-4} (Part B) or 2×10^{-5} (Part A), and two (Part A) or four (Part B) 0.1 ml samples spread on the surface of blood-agar plates (Medium 1 + horse blood + agar). Average cell counts and standard deviations given above. Differences between the averages are not significant by *t* test.

of 0.01 γ /cc of TP SIII-N was studied in media containing 0.15 per cent and 0.3 per cent agar. The consequence of decreasing the agar concentration is a threefold increase in the frequency of colonies containing transformed cells. The difference between the frequencies at the two agar concentrations is over six times the standard error of the difference and is therefore statistically significant. The mechanism by which agar concentration acts is not known, but it may conceivably operate by increasing the rate of diffusion of the factors essential to transformation. In any case, it is clear that under the standard conditions adopted for titration of TP the rate of transformation is limited by the concentration of agar.

In Table II the results of similar experiments using TP SIII-2 are also recorded. The same general result is obtained with this TP as was obtained with TP SIII-N.

It appears, therefore, that some of the scatter in the TP titration curve obtained under standard conditions with a given batch of transformation medium may be due to small, uncontrollable variations in agar concentration.

TABLE II

Effect of Agar Concentration upon Frequency of Transformations.

A. Obtained with TP SIII-N.

Expt No.	TP Conc.	0.15 per cent Agar		0.3 per cent Agar	
		Proportion Colonies Transf.	f_N	Proportion Colonies Transf.	f_N
132	0.01 γ /cc	36/60	0.600	13/60	0.216
133	0.01 γ /cc	42/56	0.745	15/59	0.254
		78/116	0.672	28/119	0.235

Difference between proportions = $0.672 - 0.235 = 0.437$.

Standard error of difference = 0.065.

B. Obtained with TP SIII-2.

Expt No.	TP Conc.	0.15 per cent Agar				0.3 per cent Agar			
		Prop. Colonies Transf.		f_2	f_N	Prop. Colonies Transf.		f_2	f_N
		Autogenic	Allogenic			Autogenic	Allogenic		
84	0.015 γ /cc	18/90	23/90	0.200	0.255	7/79	12/79	0.088	0.152
85	0.015 γ /cc	45/92	16/92	0.488	0.174	8/96	3/96	0.083	0.031
87	0.015 γ /cc	30/120	17/120	0.250	0.141	7/120	13/120	0.058	0.108

Washed agar. The effect of agar concentration suggested that materials in Difco-agar itself may be inhibitory to transformation. Agar washed according to the procedure of Ryan (9) was therefore tested in several experiments. Instead of increasing the frequency of transformation, the use of washed agar consistently reduced the frequency of colonies containing either autogenic or allogenic transformations. However, in different experiments the per cent decrease due to the use of washed agar was not uniform. The most striking effects were observed in the batches of Medium 3 which gave comparatively poor results in supporting transformation even with unwashed agar. It appeared in general that whenever conditions were poor for transformation washed agar had a pronounced effect in further reducing the frequency of transformation.

The pH of the medium. A pH range of 7.6 to 7.8 was adopted for standard conditions because it was found that when the pH of the transformation medium is below 7.2 the frequency of transformations decreases and falls

TABLE III

Influence of pH on Autogenic and Allogenic Transformations.

Final TP Concentration = 0.229 γ /cc; Agar Concentration = 0.3 per cent.

pH of Medium	6.15		6.70		7.05		7.25		7.65	
	Prop. Col. Transf.	f	Prop. Col. Transf.	f	Prop. Col. Transf.	f	Prop. Col. Transf.	f	Prop. Col. Transf.	f
Total . .	0/33	0.000	0/33	0.000	10/33	0.303	30/33	0.909	28/33	0.848
Allogenic	0/33	0.000	0/33	0.000	8/33	0.242	17/33	0.515	16/33	0.484
Autogenic	0/33	0.000	0/33	0.000	6/33	0.181	27/33	0.818	28/33	0.848

TABLE IV

Requirement of Albumin for Capsular Transformations.

A. TP SIII-2.

Final TP Concentration = 0.153 γ /cc; Agar Concentration = 0.3 per cent.

	Neither Albumin nor Pyrophosphate Added		Albumin Present; Pyrophosphate Absent		Albumin and Pyrophosphate Added	
	Prop. Col. Transf.	f	Prop. Col. Transf.	f	Prop. Col. Transf.	f
Total	16/57 ¹	0.280	54/60	0.900	52/60	0.866
Allogenic . .	10/57 ¹	0.175	24/60	0.400	28/60	0.466
Autogenic . .	8/57 ¹	0.140	50/60	0.833	46/60	0.766

B. TP SIII-N.

Final TP Concentration = 0.12 γ /cc; Agar Concentration = 0.3 per cent.

Frequency of Colonies Containing Transformed Cells.

No Albumin Added		0.1 Standard Conc. Albumin		0.5 Standard Conc. Albumin		Standard Conc. Albumin	
1/30 ¹	0.033	29/29	1.00	30/30	1.00	30/30	1.00

to zero at pH 6.7 or less (Table III). Hotchkiss (personal communication) has observed a similar effect of pH in liquid cultures. The effect of pH does not seem to be on the growth rate since colonies formed at pH 7.0 contain the same number of cells as colonies formed at a higher pH.

¹ In general, there are much fewer transformed cells per sample in colonies transformed in absence of added albumin.

Albumin and pyrophosphate concentration. A serum factor has long been known to be necessary for the induction of capsular transformations (1). It was later shown that this factor is albumin (6). The requirement for serum albumin is clearly demonstrated by the results of experiments summarized in Table IV. In media without added albumin the frequency of transformations is considerably lower than that observed in the presence of albumin. The few transformations that are occasionally observed in the absence of added albumin may be explained by the fact that Medium 3 contains an extract of beef serum which may be a source of small amounts of albumin. The amount of albumin utilized under standard conditions is apparently greatly in excess of that required for maximal incidence of transformation (Table IV B).

Sodium pyrophosphate was found to be effective in reactivating serum which had been inactivated by dialysis (7). For this reason, sodium pyrophosphate is often added as a standard constituent of transformation medium. From the experiments summarized in Table IV A, it appears that, under the conditions in which the present experiments were performed, pyrophosphate is not essential for transformation since the frequencies of autogenic and allogenic transformations are the same in the absence or presence of this substance.

Comparison of autogenic and allogenic transformations

TP SIII-1c

This TP induces allogenic transformations of strain SIII-1 to SIII-N. Since SIII-1c cells are indistinguishable morphologically from SIII-1 cells,

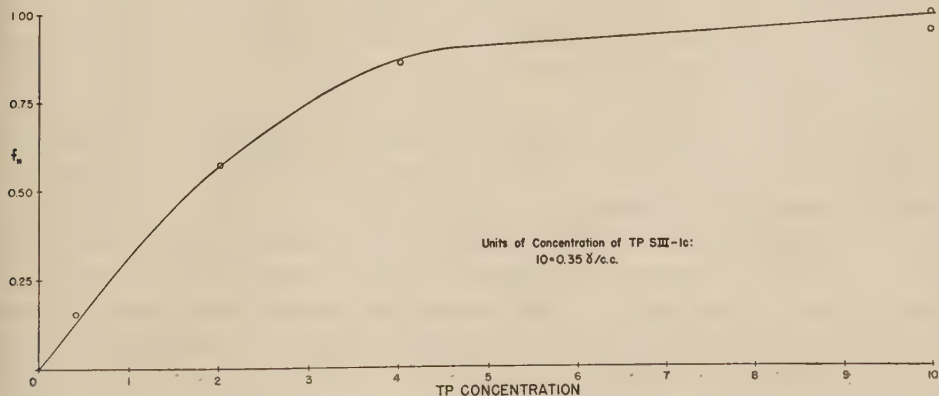


Fig. 5. Frequency of SIII-1 colonies containing allogenic transformations (f_N) as a function of concentration of TP SIII-1c.

it is not technically feasible to determine whether it induces at the same time any autogenic transformations to SIII-1c. The frequency of SIII-1 colonies containing SIII-N cells as a function of the concentration of TP SIII-1c is shown in Figs. 5 and 6. It can be seen that the linear relation previously observed between concentration of TP and frequency of autogenic transformations holds as well for allogenic transformations. It should also be

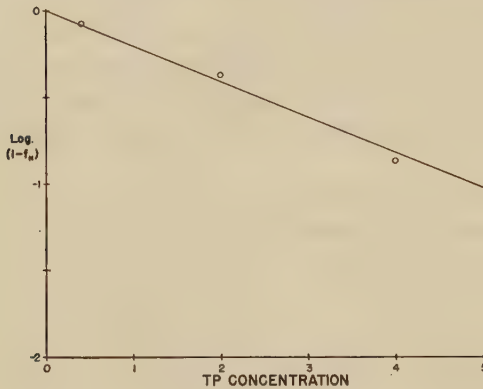


Fig. 6. Logarithm of frequency of SIII-1 colonies containing no allogenically transformed cells ($1-f_N$) as a function of concentration of TP SIII-1c.

noted that every SIII-1 colony contains cells capable of allogenic transformation, for at sufficiently high concentrations of TP SIII-1c, all colonies contain transformed cells.

TP SIII-2

Independence of autogenic and allogenic transformations. The relation between the concentration of TP SIII-2 and the frequency of SIII-1 colonies containing autogenically transformed (SIII-2) cells was examined above. The justification for the separate examination of autogenic and allogenic transformations induced by this TP is based on the finding that the two types of transformation occur independently of each other. The results of a large number of experiments are given in Table V in which are recorded the the total frequency of colonies containing autogenic transformations (f_2), the total frequency of colonies containing allogenic transformations (f_N), and the frequency of colonies in which both types of transformations were found (f_{2+N}). If autogenic and allogenic transformations took place independently of one another, the frequency of colonies containing both kinds of transformed cells (f_{2+N}) should be equal to the frequency of colonies containing allogenic transformations (f_N) multiplied by the frequency of colonies con-

TABLE V

Frequency of SIII-1 Colonies in which Autogenic and Allogenic Transformations Occur as a Function of Concentration of TP SIII-2.

TP Conc. (γ /cc)	No. of Colonies Isolated	Total No. of Col. with Transf. (T)	f_T	No. with Allo. Transf. (N)	f_N	No. with Auto. Transf. (2)	f_2	No. with Both Types (N+2)	Observed f_{N+2}	Ex-pected f_{N+2} ($f_N \times f_2$)
0.458	30	30	1.000	19	0.633	29	0.966	18	0.600	0.611
	30	30	1.000	21	0.700	30	1.000	21	0.700	0.700
0.305	30	30	1.000	21	0.700	28	0.933	19	0.633	0.653
	30	30	1.000	18	0.600	29	0.966	18	0.600	0.579
0.229	66	58	0.878	33	0.500	55	0.833	30	0.454	0.416
	40	39	0.975	32	0.800	39	0.975	32	0.800	0.780
0.183	39	39	1.000	30	0.769	34	0.871	27	0.641	0.670
0.153	39	32	0.820	17	0.435	29	0.743	14	0.358	0.323
	120	107	0.891	52	0.433	96	0.800	42	0.350	0.346
0.091	89	78	0.879	56	0.617	61	0.685	39	0.438	0.423
	30	27	0.900	19	0.633	24	0.800	16	0.533	0.506
	58	49	0.844	30	0.517	43	0.741	24	0.413	0.383
	59	46	0.779	30	0.508	33	0.559	17	0.288	0.284
	60	54	0.900	33	0.550	46	0.766	25	0.416	0.421
	70	46	0.657	19	0.271	36	0.514	9	0.128	0.139
0.078	60	43	0.716	27	0.450	34	0.566	18	0.300	0.254
0.061	60	39	0.650	22	0.366	29	0.483	12	0.200	0.177
	40	23	0.575	13	0.325	18	0.450	8	0.200	0.146
	70	49	0.700	27	0.385	38	0.542	16	0.228	0.209
	75	74	0.986	57	0.760	47	0.626	30	0.400	0.475
0.057	63	41	0.650	21	0.333	34	0.539	14	0.222	0.179
0.045	73	39	0.534	18	0.246	27	0.369	6	0.082	0.091
	70	37	0.528	15	0.314	15	0.314	7	0.100	0.099
	30	15	0.500	8	0.266	9	0.300	2	0.066	0.079
	59	28	0.474	19	0.322	20	0.338	11	0.186	0.108
	73	40	0.547	22	0.301	32	0.438	14	0.191	0.132
	90	62	0.688	39	0.433	36	0.400	13	0.144	0.173
0.038	67	41	0.611	33	0.499	22	0.328	14	0.208	0.164
0.030	100	49	0.490	35	0.350	24	0.240	10	0.100	0.084
	60	30	0.500	18	0.300	17	0.283	5	0.083	0.085
0.022	87	34	0.390	23	0.264	19	0.218	8	0.092	0.057
	75	23	0.306	15	0.200	11	0.146	3	0.040	0.039

(Table V Cont.)

TP Conc. (γ/cc)	No. of Colonies Isolated	Total No. of Col. with Transf. (T)	f_T	No. with Allo. Transf. (N)	f_N	No. with Auto. Transf. (2)	f_2	No. with Both Types ($N+2$)	Observed f_{N+2}	Expected f_{N+2} $f_N \times f_2$
0.015	53	17	0.320	14	0.264	4	0.075	1	0.019	0.020
	79	16	0.202	12	0.151	7	0.088	3	0.037	0.013
	96	11	0.114	3	0.031	8	0.083	0	0.000	0.002
	120	20	0.166	13	0.108	7	0.058	0	0.000	0.005
0.012	120	27	0.225	17	0.141	12	0.100	2	0.016	0.014
0.011	75	17	0.226	12	0.160	8	0.106	1	0.013	0.017
0.007	73	19	0.260	17	0.232	2	0.027	0	0.000	0.006
	138	34	0.246	28	0.202	18	0.130	4	0.029	0.026
0.006	100	17	0.170	11	0.110	8	0.080	1	0.010	0.008
	120	13	0.108	4	0.033	9	0.075	0	0.000	0.002
0.004	150	21	0.140	17	0.114	5	0.033	1	0.006	0.003
0.003	148	10	0.067	6	0.040	4	0.027	0	0.000	0.001

Test of independence of obs. and exp. f_{N+2} .

Combined χ^2 = 53.21 (= 27.28 omitting experiments in which the frequency of any class is less than five.)
 Degrees of Freedom = 43 (= 20).
 P = > 0.1 and < 0.2 (= 0.12).

taining autogenic transformations (f_2). The expected frequencies of colonies containing both types of transformations calculated on this assumption are given in the final column of Table V. It is clear that at all concentrations of TP the observed f_{2+N} is very close to that expected if autogenic and allogenic transformations were independent events (combined $\chi^2_{43} = 53.21$; $P = > 0.1$ and < 0.2). It may be concluded, therefore, that the occurrence of one type of transformation is not dependent upon the occurrence of the other type.

It is evident that the frequency of colonies containing both SIII-2 and SIII-N cells depends upon the concentration of TP in the medium: the higher the concentration of TP employed, the greater the over-all frequency of colonies containing transformed cells of either type and the greater the frequency of colonies containing both types of transformations. Autogenic and allogenic transformations being independent events, it is evident that the frequency of multiple "hits" (several individual cell transformations) per colony increases with TP concentration. This is, of course, consistent

with our interpretation of the shape of the curves relating concentration of TP to the frequency of colonies containing transformed cells: the deviation from linearity at high TP concentration is assumed to be due uniquely to the occurrence of multiple "hits".

Limitation of maximal frequency of allogenic transformations. The frequency of allogenic transformations as a function of the concentration of

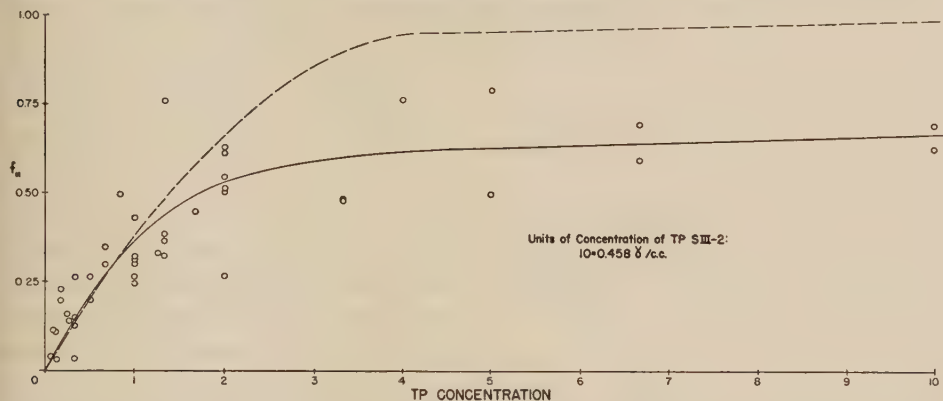


Fig. 7. Frequency of SIII-1 colonies containing allogenic transformations (f_N) as a function of concentration of TP SIII-2. Each point represents a measurement obtained in a single experiment in which several concentrations were usually tested (see Table V). The dashed line, representing the curve obtained for autogenic transformations measured in the same experiments (Fig. 1), is presented for the purpose of comparison.

TP SIII-2 is shown in Fig. 7. The curve relating the frequency of autogenic transformations to TP concentration is drawn in a dotted line on the same graph, for the purpose of comparison. A linear relation seems to hold between the frequency of colonies containing allogenicly transformed cells and low concentrations of TP. However, because the scatter of the data is so much greater than that observed for autogenic transformations and because an extremely large number of colonies need to be examined to obtain precise data at low frequencies, it is not possible to determine the exact kinetics of allogenic transformation. Nevertheless, it is clear from the data that within the range of concentrations of TP at which there is a continuous linear increase in the frequency of autogenic transformations, the rate of increase of the frequency of allogenic transformations induced by TP SIII-2 slows down abruptly and attains asymptotically a value of ca. 0.70. This must mean that under the experimental conditions some factor other than concentration of TP limits the maximal frequency of colonies containing SIII-N cells.

TABLE VI

Effect of Agar Concentration upon Maximal Frequency of Colonies in which Allogenic Transformations are induced by TP SIII-2.

Expt No.	TP Preparation No. and concentration	0.15 per cent Agar		0.3 per cent Agar	
		Prop. Colonies containing Allogenic Transf.	f_N	Prop. Colonies containing Allogenic Transf.	f_N
118	No. 5—0.153 γ /cc	47/50	0.940	52/120	0.433
117	No. 5—0.183 γ /cc	65/68	0.955	30/117	0.256
134	No. 6—3.84 γ /cc	23/23	1.000	25/30	0.833

Fig. 2 B presents a semi-log plot of the frequency of colonies in which no allogenic transformations are induced as a function of the concentration of TP SIII-2. It may be remarked that the relation between allogenic transformations and TP concentration is probably linear up to the point where 40 per cent of the colonies contain allogenicly transformed cells. Thereafter some factor other than concentration of TP limits the rate of increase of the frequency of allogenic transformations.

The role of agar concentration in altering the frequency of transformations has already been mentioned (Table II). It was found that the concentration of agar in the transformation medium also influences the maximum level of allogenic transformations that can be attained. Decreasing the agar concentration to 0.15 per cent permits allogenic transformations to occur in all of the colonies (Table VI). Thus, it seems possible that agar concentration, perhaps by limiting the rate of diffusion of some essential substance in the medium, controls at once the rate of increase of transformation with TP concentration and the highest attainable frequency of SIII-1 colonies in which SIII-N transformations can be induced by TP SIII-2.

It is clear from Figs. 3 and 5 that, in 0.3 per cent agar, transformations to SIII-N can occur in all of the colonies when the transforming agent is either TP SIII-N or TP SIII-1c. When TP SIII-2 is used, however, transformations to SIII-N can occur in only 70 per cent of the colonies. This limitation cannot be attributed to the fact that the transformations to SIII-N induced by TP SIII-2 are allogenic, for the frequency of allogenic transformations to SIII-N is not limited when the TP used is SIII-1c (Fig. 5). The same limitation has been observed with a second preparation of TP SIII-2, extracted and purified two years later, so that it does not seem likely that the results obtained can be due to ageing of the TP or to a particular preparation.

GENERAL DISCUSSION

A practical result of this study is the development of a method which can be utilized in the quantitative assay of capsular transformations. The advantages of this method are obvious when, for example, one wishes to titrate transformations as a function of physical or chemical conditions of the medium. The disadvantages reside chiefly in the fact that large numbers of colonies must be isolated and examined to obtain significant results when the effect being studied is small.

There are several shortcomings to the method, however, from the point of view of interpretation of the kinetics of transformation. A number of assumptions need to be made, only a few of which can be directly tested. One of the most important of these assumptions is that the time during which transformations can take place within a given clone is governed and limited by the very nature of the biological system involved. This assumption was based on the experiments of McCarty, Taylor and Avery (7) with liquid cultures which showed that TP added after a fairly brief period of growth does not induce any transformations. The loose gel of the semi-solid medium makes it technically impossible to perform similar experiments in this system, but it was expected that the same limited period of "sensitization" would operate in semi-solid media. In any case, the study of only a small sample of each colony formed in the semi-solid medium serves to assure that only transformations occurring within a controlled period of time are scored. That transformations are still occurring close to the time limit imposed by this method is suggested by the fact that some samples of colonies exposed to low concentrations of TP contain as few as one or two transformed cells. It is possible that a delay of several generations takes place before the effective contact between TP and bacterium results in the transformation of *one* of the daughter cells. Alternatively, it is possible that the transformed cells are at a selective disadvantage during the growth of the colony: a growth rate slower than that of the untransformed cells might account for a proportion of transformed cells smaller than would be expected on the basis of the time of occurrence of the transformation in the clone. The only way to discriminate between these possibilities would be to study the changes occurring in individual colonies as a function of time. This would obviously be a large undertaking, for it would require the examination of many colonies exposed to different concentrations of TP for varying periods of time. The results of such a study would be worthwhile, however, in furnishing some idea of the time in which transformations occur in colonies growing in semi-

solid media and in measuring the role of selection as a factor determining the proportion of transformed cells in a clone.

Since TP can only be added to a semi-solid medium before the gel is set, TP must be present during the entire time the bacteria are becoming sensitized. The extent to which TP decays in semi-solid medium is not known. If a decay were at all appreciable, the effective concentration of TP at the earliest time at which transformations can occur is obviously the initial concentration less the concentration inactivated during the sensitization period. This would mean that the effective concentration is less than the initial one. A more serious problem from the point of view of kinetic interpretation is the possibility that TP continues to decay during the transformation period, so that decreases in TP concentration are not solely a reflection of the amounts taken up by transformed bacteria. Furthermore, it is important to know if the rate of TP decay is a function of initial TP concentration. This possibility would, of course, enormously influence the kinetic picture of transformation.

The conditions for a clear interpretation of the kinetics of transformation in a semi-solid medium are, therefore, still far from satisfactory. While one might hope to improve the situation by studies of TP decay and of the changes occurring with time in single colonies, the results already obtained are sufficiently encouraging to warrant a tentative interpretation. The frequencies plotted in Figs. 1, 3, 5 and 7 are obviously not the frequencies of cell transformations, for a colony containing transformed cells may have been one in which one, two or more individual cell transformations occurred. One can overcome this difficulty, however, by examining the logarithm of the frequency of "untransformed" colonies as a function of the concentration of TP. In this way, one can correct, so to speak, for the deviations from linearity which are due solely to the increasing frequency of multiple "hits". The linear relation between the frequency of transformations and the concentration of TP obtained in this way is taken to mean that the effective contact between one sensitized pneumococcus and one unit (molecule?) of the specific Type III transforming agent results in the transformation of the bacterium.

If a linear relation between the frequency of transformation and concentration of TP were a general characteristic of transforming systems, one should expect to be able to verify this finding on a cellular level by a kinetic study of transformation to a character such as antibiotic resistance. For in such a system it is possible to determine directly the number of bacteria transformed as a function of concentration of TP because the problem

of "screening" the transformed from the untransformed cells is solved by the selective elimination of the untransformed cells. A study by Hotchkiss (personal communication) indicates that a linear relation between transformation and TP concentration holds for transformations to streptomycin resistance. Stocker, Krauss and MacLeod (9) have also recently reported a linear response of transformations of "rough" pneumococci to change in concentration of "smooth" transforming agent.

One of the most clear-cut results of the present investigation is the establishment of the independent occurrence of autogenic and allogenic transformations in a system where both take place. One of the most important implications of this finding is that the transformation from SIII-1 to SIII-2 is not an intermediate step in the transformation to SIII-N induced by TP SIII-2, nor conversely is the transformation to SIII-N an intermediate step in the transformation to SIII-2. The two types of transformation induced by TP SIII-2 in SIII-1 cells are distinct, separate events probably involving different mechanisms. To account for this independence one could suppose that in preparations of TP capable of inducing allogenic transformations, there are actually two different agents affecting the synthesis of Type III polysaccharide: one agent inducing the allogenic transformation, the other inducing the normal autogenic transformation. Such an hypothesis is untenable unless one makes a variety of supplementary assumptions to explain why TP SIII-2 induces uniquely autogenic transformations with the rough (R) strain of pneumococcus. This difficulty is further illustrated by the following case observed by Ephrussi-Taylor (3): the TP extracted from mutant strain SIII-1a (indistinguishable morphologically and serologically from strain SIII-1) transforms R cells to SIII-1a (autogenically), transforms SIII-1 cells to SIII-2 (allogenically), and transforms SIII-1c cells to SIII-N (allogenically). A "multi-agent" hypothesis would require that TP SIII-1a contain three agents, an SIII-1a agent which can act only upon R cells, an SIII-2 agent which can act only upon SIII-1 cells, and an SIII-N agent which can act only upon SIII-1c cells. These three agents must be assumed, furthermore, to differ from the agents which are found respectively in the SIII-1a, SIII-2 and SIII-N strains produced by transformation, for agents from all three transformed strains can act upon either R, SIII-1 or SIII-1c cells.

It seems more economical to suppose that the agent inducing allogenic transformations is the same as the one inducing autogenic transformations, that the autogenic transformation is the outcome of a replacement by this agent of the one already existing in the bacterium (11, 12) and that the al-

logenic transformation is the result of an interaction between these two agents constituting a new agent (3). If autogenic and allogenic transformations are induced by the same agent, how then can one account for the different shapes of the curves relating the frequencies of autogenic and allogenic transformations to the concentration of TP SIII-2? It has been shown that while autogenic transformations can be induced by TP SIII-2 in all of the colonies grown in 0.3 per cent agar, allogenic transformations can take place in only about 70 per cent of these colonies. One could imagine that in 30 per cent of the colonies grown in medium containing a high concentration of TP SIII-2 the autogenically transformed cells somehow exclude the possibility of any allogenic transformations occurring in the same clones. This hypothesis is, however, inconsistent with the finding of independence between the frequencies of colonies containing autogenic and allogenic transformations. Such a result could not have been observed had there been any strong selective effect exerted by cells transformed to SIII-2 against subsequent allogenic transformations in the same clone.

Our present working hypothesis assumes that all colonies contain cells capable of undergoing this particular allogenic transformation (or a sufficiently large number of cells capable of allogenic transformation to insure at least some transformations taking place during the period of "sensitization"). The working hypothesis further assumes that the concentration of some factor in the medium not only limits the rate of increase of frequency of transformation as a function of TP concentration but also determines the maximal proportion of colonies in which this kind of allogenic transformation can take place. Decreasing the agar concentration and, hence, the viscosity of the medium increases the rate of diffusion of this factor to (or from) the growing colonies and thereby increases the proportion of those in which allogenic transformations to SIII-N can occur. The physiological state of the bacterium, as controlled through the environment, is thus assumed to play an important role in determining the outcome of the contact between bacterium and unit of TP.

It is still rather puzzling why the limitation of the frequency of colonies containing allogenic transformations to SIII-N should be peculiar to TP SIII-2 inasmuch as it is not observed with TP SIII-1c. For the moment one can only suppose that this is due to specific properties of the SIII-1/SIII-2 pair of transforming agents which are not shared by the SIII-1/SIII-1c pair. Perhaps the reconstitution of the SIII-N agent from the interaction of SIII-1 and SIII-2 agents is more sensitive to the hypothetical environmental factor than the one resulting from the interaction of SIII-1 and SIII-1c agents.

It would be interesting to utilize the method described in this paper for the purpose of a quantitative comparison of different transforming agents capable of effecting similar transformations. For example, what difference in efficiency is there, if any, between TP SIII-2 and TP SIII-1c in inducing allogenic transformations from SIII-1 to SIII-N? Unfortunately, so long as it is impossible to gauge the quantity of biologically inactive TP which may still give a positive Dische reaction, it will remain impossible to make comparisons of efficiency between different TP preparations solely on the basis of the amount of Dische-positive material contained in them.

SUMMARY

1. Pneumococci of strain SIII-1 may undergo capsular transformations when grown as colonies suspended in semi-solid agar medium containing the purified transforming agent extracted from other strains capable of synthesizing the Type III capsular polysaccharide. The frequency of colonies containing transformed cells can be measured as a function of a given variable.

2. The logarithm of the frequency of colonies containing no transformed cells plotted as a function of the concentration of transforming agent in the medium gives a straight line intercepting the ordinate axis at a value representing 100 per cent of the colonies. This finding is in accordance with the hypothesis that one unit of transforming agent (TP) reacting with one sensitized pneumococcus is sufficient to transform the bacterium.

3. The influence of several environmental factors has been studied. The concentration of agar, hydrogen ions and albumin significantly influence the frequency of transformations. In addition, certain unknown factors in the basal medium employed in transformation studies appear to affect the rate of increase of transformation frequency as a function of the concentration of TP.

4. When the transforming agent obtained from strain SIII-2 (TP SIII-2) is allowed to act upon growing colonies of strain SIII-1, autogenic transformations occur in some of the colonies, allogenic transformations in others, and both types of transformation occur in a small proportion of the colonies. The frequency of colonies containing both types of transformed cells is that expected if autogenic and allogenic transformations are discrete events occurring independently of each other.

5. In a standard semi-solid transformation medium (containing 0.3 per cent agar) autogenic transformations can occur in all of the colonies. This

is true whether the inducing agent is TP SIII-N or TP SIII-2. Allogenic transformations can also occur in all of the colonies when the inducing agent is TP SIII-1c. When TP SIII-2 is used, however, allogenic transformations are limited to about 70 per cent of the colonies. This limitation is removed if the agar concentration is decreased, in which case allogenic transformations can be found in all of the colonies.

6. An hypothesis is presented according to which the physiological state of the pneumococcus, as controlled by the environment, determines the nature of the transformation induced by a given unit of capsular transforming agent.

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CHANGES IN NUCLEIC ACIDS OF CELLS OF MOUSE MAMMARY CARCINOMA FOLLOWING EXPOSURE TO X-RAY OF LOW DOSAGE¹

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MUCH of experimental work on the effect of radiations on tissues, both *in vivo* and *in vitro* has been carried out with radiations of comparatively high dosage. Strangeways and co-workers (9, 10) early studied the effect on mitosis of small doses of X-ray, namely 50 to 150 roentgens, in rapidly growing cultures of cells from chick embryo and observed an initial temporary inhibition of mitosis followed by recovery with a return to, or slightly above, the pretreatment level of mitotic rate. These mitotic changes occurred during a period of about four hours following X-radiation and the values obtained could be demonstrated graphically as a graded series of curves, directly related to the dosage of X-ray used. The findings of Strangeways were confirmed and amplified by other workers especially Lasnitzki (3). A considerable literature has accumulated and one of the most recent papers on the subject is that of Friedenwald and Sigelman (2). If cell division, as is believed, is associated with a rise in nucleic acids of the cell, then a correlation between changes in mitotic rate and cellular nucleic acid should exist. Experiments outlined in this paper were an attempt to follow the changes in nucleic acids of a cellular suspension of mouse mammary carcinoma in Ringer's solution following exposure to X-ray of low dosage.

MATERIALS AND METHODS

Two transplantable mouse mammary carcinomas, EO771 and b₁, which were maintained in C₅₇ BL. mice were used in this study. Both tumors were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Tumor cells were inoculated intraperitoneally, but at no time did an ascites tumor de-

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velop. The intraperitoneal implants when removed in 10 to 14 days after inoculation extended along the mesentery in widespread small nodules with little or no hemorrhage and necrosis. These growths were used in the production of a cellular suspension in Ringer's solution. Details for obtaining a discrete uniform cellular suspension were similar to those already described (5). Method for analysis of cellular nucleic acids was identical with that previously outlined (4).

During the different procedures employed in the production of a uniform discrete cellular suspension, the tubes containing the tumor tissue and cellular suspension were kept packed in crushed ice. As soon as the cells in the filtered suspension were found to be uniformly distributed, a cell count per cmm was made in a Neubauer hemocytometer. Then, 1 ml was removed for the initial analysis of desoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) per cell. The remaining solution was transferred to a thin-walled plastic vial of about 10 ml capacity with a screw top. The vial containing the suspension was at once irradiated with 400 KV X-rays with a half-value layer of 3.95 mm copper. The target-object distance was 50 cm. The X-ray dose to the vial was controlled by placing a "Victoreen Integron" dosimeter in a parallel position in the beam in air. Both the vial and the ionization chamber were supported free in air. Secondary scatter was negligible and ignored. The rate of irradiation at the vial was 20 roentgens per minute number of roentgens (r.) delivered to a sample were 75, 100 or 150. Immediately after irradiation, the contents of the vial were pipetted in 1 ml amounts into glass tubes which had been chilled by packing in crushed ice. The cell count at the time of separation of the suspension did not appreciably differ from that of the original non-irradiated sample and was used as a basis for calculation of cellular changes in nucleic acids found by chemical analysis at successive intervals following X-radiation. The separation of the irradiated suspension into individual samples for analysis was instituted because of morphologic and chemical changes which developed in many of the cells following X-radiation and which rendered their enumeration difficult. In Giemsa-stained films many of the cells showed within the cytoplasm 1 to 5 small blue staining foci with a denser stained compact center. Frequently these foci developed as globular extrusions at the periphery of the cell and in the manipulations used in cell enumerations some of them became detached and circulated as free small blue masses. The formation of the foci varied in time in different cells, in a few of which the process began shortly after irradiation. The masses varied in size, averaging about 1μ in diameter. A separate tube, containing less than 1 ml of irradiated suspensions was kept under the same conditions as the other tubes, and was used for making films from time to time. The individual tubes containing the irradiated suspensions were kept at 4°C temperature and at varying intervals one was removed for chemical analysis of the contents. As controls for autolytic changes at low temperatures which may also be taking place in the nucleic acids of these suspensions we have used values shown by us to be small in amount and related to the total content of DNAP and RNAP initially present in the cell. At 4°C these changes were negligible. The amount of available suspension determined the number of analyses and the sampling intervals. The intervals had a range of 30, 45, 60, 90, 120, 180 and 240 minutes. In later experiments the shorter intervals were selected because of the observed rapidity of change in the nucleic acids shortly after X-radiation of 100 r.

RESULTS

The results are arranged in Table I in Groups I, II, and III. In each group the data are aligned with cellular DNAP obtained in the non-irradiated suspension of cells in ascending order of values except for No. 5 in Group I. The results showed wide variations, depending on the dosage of X-ray and also on the content of cellular nucleic acid at the time of irradiation. In Group I, the dosage of X-ray was 75 r. and 150 r. and throughout Groups II and III, dosage was 100 r.

A dose of 150 r. employed in Experiment No. 5, Group I, induced a continuous fall in the nucleic acids and was not repeated. The two ways by which reduction in nucleic acids may occur are illustrated in Fig. 1, on data plotted from Experiment No. 5. In this figure, the abscissa is $\text{DNAP} \times 10^{-9}$ mg and ordinate is $\text{RNAP} \times 10^{-9}$ mg per cell and related pairs of nucleic acids are plotted as points. The first reduction was a perpendicular downward migration of RNAP alone along the line of DNAP at 1.20×10^{-9} mg per cell and the second reduction, including both DNAP and RNAP, was a fall along the line of regression with a coefficient of 0.68. X-radiation of 75 r. in Experiments Nos. 2, 3 and 4 produced little change in cellular nucleic acid except at 1 or more hours. Changes occurring in Nos. 2 and 4 involving the RNAP are shown in Fig. 2. The two different types of reaction taking place in the increase or decrease of nucleic acid in a cell are graphically recorded in Fig. 3, from data obtained in Experiment No. 3. The changes in the first 30 minutes are within the limits of technical error. Subsequent decrease to an implied constant level of DNAP with fluctuating values of RNAP probably are an index of the changes taking place because alkaline hydrolysis for 12 and 18 hours on separated halves of the residue containing the total nucleic acid gave comparable values at 1.5, 2 and 3 hours. The extent of the rise in RNAP along the line of DNAP at 1.00×10^{-9} mg per cell was considerable, and attention is called to the accompanying high RNAP/DNAP ratio, a feature whose definitive activity in developing nucleic acid increment will be pointed out in Groups II and III.

The remaining experiments, carried out at an exposure of 100 r., were divided into 2 groups based on the timing selected for the sampling intervals. In Group II (Nos. 20-25), the sampling intervals extended over 3 or 4 hours, and were more widely separated than in Group III (Nos. 26-32). In Group III, the last sampling interval was at 2 hours following irradiation. During these two hours 4 or 5 determinations of nucleic acids were carried out. This arbitrary division into Groups II and III with change in the timing

Changes in cellular nucleic acids following X-irradiation

No. Group of Expt.****	0 min.**		30 min.		45 min.		60 min.		90 min.	
	DNAP	RNAP	DNAP	RNAP	DNAP	RNAP	DNAP	RNAP	DNAP	RNAP
I 2	1.19	0.54			1.20	0.55				
4	1.20	0.74			1.20	0.65				
3	*1.60	1.04	1.64	1.07	1.50	1.00	1.00	1.54	0.98	1.50
									0.96	1.50
5	1.20	0.98			1.20	0.84				
II 23	0.83	0.67			1.00	0.54				
20	1.02	0.68					1.31	0.92		
21	1.06	0.60			1.20	0.73				
22	1.20	0.50					1.20	0.56		
24	*1.50	0.69					1.20	1.00		
25	1.50	0.62					1.33	0.66		

III 30	0.84	0.39	0.85	0.35	0.85	0.40	0.68	0.68	0.61	0.57
							0.68	0.50	0.67	0.50
32	1.00	0.84	1.10	1.98			0.96	0.87	0.86	0.92
							1.05	0.77	0.99	0.86
29	1.17	0.60	1.05	0.65	1.03	0.68	0.79	0.88	0.67	0.93
							0.84	0.82	0.75	0.84
31	1.20	0.60	1.20	0.62			0.96	0.87	0.86	0.92
							1.05	0.77	0.99	0.86
27	1.20	0.84	1.33	1.11	1.35	1.13	1.20	0.94	1.08	1.00
									1.15	0.90
26	*1.50	0.90	1.60	1.37	1.50	0.86	1.50	0.84	1.36	1.00
28	1.50	0.77	1.50	0.59	1.50	0.90	1.50	0.90	1.30	0.86
							1.37	0.84	1.34	0.86

* Mouse mammary tumor used in these 3 experiments was b_1 , that used in all other experiments was E 0771. Both were grown in C_{57} black mice.

** DNAP and RNAP values listed are expressed in 10^{-9} mg per cell.

*** Primary separation of RNAP from the residue containing total nucleic acid by hydrolysis with N KOH at 37°C . was carried out in duplicate in Group III only in no. 26. In the other 6

of intervals in Group III, was due to the observation that as analytic values for nucleic acids accumulated in Group II, at or after 1 hour following irradiation, an anomalous and unexpected gradual increase in RNAP, as-

cellular suspension of mouse mammary carcinoma.

0 min.		160 min.		3 hrs.		4 hrs.		24 hrs.		RNAP/DNAP ratio of analysis of control suspension
P	RNAP	DNAP	RNAP	DNAP	RNAP	DNAP	RNAP	DNAP	RNAP	
		1.20	0.69							0.45
		1.20	0.83			1.30	0.89	(?)		0.61
0	1.40			1.04	1.25					0.65
0	1.36			1.04	1.22					
		0.95	0.62			0.90	0.54			0.82
5	0.63			1.20	1.52	0.90	0.61			0.80
2	0.65					1.04	0.69			0.67
6	1.46			1.20	0.69	1.10	0.60	0.98	0.55	0.56
7	0.52			1.12	0.56	1.06	0.52			0.42
0	0.89			1.12	0.75					0.46
0	0.77			1.10	0.80					0.41
1	0.68	N KOH hydrolysis for 18 hours								
5	0.63					6				0.46
4	0.77					18				
2	0.89					6				0.84
3	0.91					18				
9	0.86					12				0.51
4	0.97					18				
2	0.89					6				0.50
1	0.92					18				
0	0.78					12				0.70
5	1.40					18				0.60
8	1.04					18				
8	0.83					12				0.51

experiments of Group III, duplicate analyses after 18 hours hydrolysis were replaced by an analysis on one half of the residue after 18 hours hydrolysis, and an analysis on the second half of the residue after hydrolysis for the time (6 or 12 hours), stated in the table.

**** Dose of X-ray in r., Group I, Experiments Nos. 2, 3 and 4 was 75 r. and for No. 5 was 150 r. In Groups II and III throughout dose of X-ray was 100 r.

sociated with a fall in DNAP took place, and will be discussed later. This anomaly was apparently related to an increased lability of DNA occurring in the primary hydrolysis, with N KOH at 37°C, of the residue containing

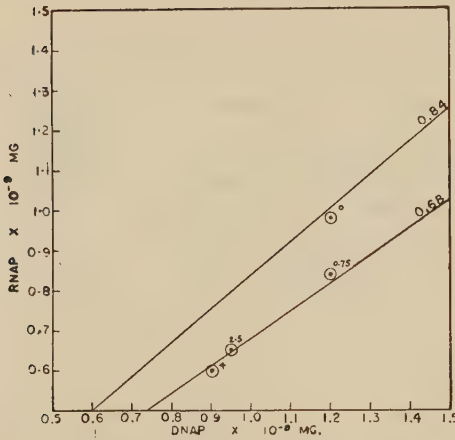


Fig. 1.

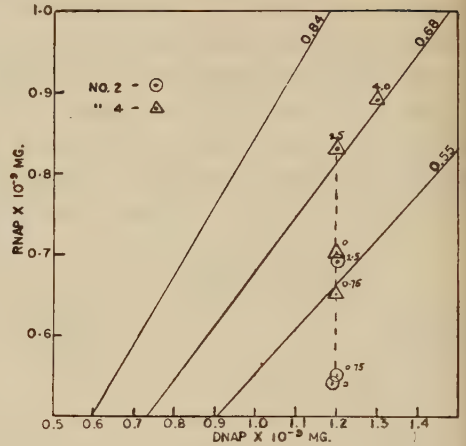


Fig. 2.

1. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 150 r. Data from Expt. No. 5 in Table I. Small figure placed on the upper right of symbols, in this and other figures, indicates the time at which sample was taken for analysis.
2. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 75 r. Data from Expts. No. 2 and 4 in Table I.

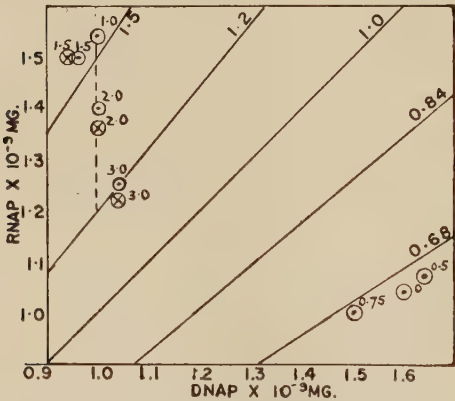


Fig. 3.

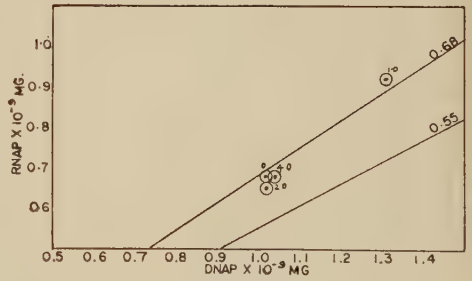


Fig. 4.

3. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 75 r. Data from Expt. No. 3 in Table I.
4. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 100 r. Data from Expt. No. 20 in Table I.

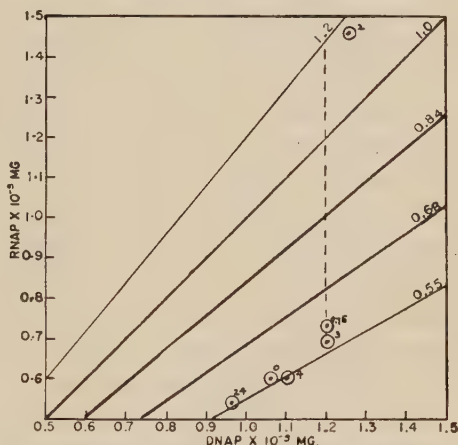
the total nucleic acids. This primary hydrolysis was designed by Schmidt and Thannhauser (6) to separate the RNA from the more resistant DNA.

In Fig. 4, plotted from data in Experiment No. 20, a rise in nucleic acids

up to 1 hour and then back over the same route to the point of origin in the next hour is shown. Fig. 5, plotted from data in Experiment No. 21, also shows an initial rise succeeded by a fall in the nucleic acids.

An interesting feature brought out by X-radiation of suspensions of tumor cells, was the relation of RNAP/DNAP ratio to post-irradiational increase in cellular nucleic acids. In Group II, exposure to 100 r. gave within 30 to

5. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 100 r. The position of the points at 3 and 4 hours appear to be higher than expected and this irregularity is probably an indication of instability of the DNA undergoing alkaline digestion. Data from Expt. No. 21 in Table I, group II.



60 minutes varying results related to the initial, non-irradiated RNAP/DNAP ratio. In three experiments, Nos. 20, 21 and 23 in which the RNAP/DNAP ratio was 0.56 or above, a rise in nucleic acid occurred. In Nos. 22, 24 and 25, in which the RNAP/DNAP ratio was 0.46 or below, practically no increase in cellular DNAP occurred. In RNAP no appreciable change occurred in No. 22 and a variable increase in Nos. 24 and 25. The relationship between high RNAP/DNAP ratio and increase in cellular nucleic acid upon X-radiation was borne out by results shown in Group III. In Nos. 26, 27 and 32 of Group III with ratios respectively of 0.60, 0.70 and 0.84, an appreciable rise in nucleic acid took place within 45 minutes after exposure to X-ray of 100 r. The remaining 4 experiments, namely Nos. 28, 29, 30 and 31 with a ratio of 0.51 or less showed little or no increase in nucleic acids to this dosage of X-ray in 45 minutes. The reaction occurring in No. 27 will be discussed later.

With an X-ray dosage of 75 r., a definitive role of high RNAP/DNAP ratio in stimulating a rise in cellular nucleic acid was not apparent. Also as seen in No. 5, Group I, despite high RNAP/DNAP ratio, the deleterious effects of 150 r. in the cell militated against any favorable influence of high ratio in stimulating a rise in nucleic acid.

Thus, with an X-ray dosage of 100 r. a rise in nucleic acids occurred in 6 of 13 experiments of Groups II and III where the initial non-irradiated, RNAP/DNAP ratio was over 0.55, and little or no rise occurred in the remaining 7 experiments, in which the RNAP/DNAP ratio was below 0.55.

As the results of chemical analysis of suspensions exposed to X-ray of 100 r. accumulated in Group II, the phenomenon already mentioned, of an anomalous increase in cellular RNAP, associated with a decrease in DNAP at 1 hour or shortly after, became apparent. The rise of RNAP was well exemplified in Experiment No. 24 and less well in Experiment No. 25. It is interesting that in these 2 experiments the augmented RNAP started only at, or shortly after, 1 hour and appeared to be related to increased lability and partial breakdown of DNAP resulting from the effects of irradiation of 100 r. In retrospect, it seems probable that had more frequent samplings and analyses been made during the first two hours throughout the experiments in Group II, the aberrant rise in RNAP might also have been recognized in other experiments of this group. The amounts of DNA and RNA obtained in our chemical analyses were calculated on the phosphorus content of the two separated nucleic acids (6) and not on the content of characteristic sugar (7, 8) in each nucleic acid, so that the mechanism of the irregular increase in RNA was not clear. Part of the increase may possibly have been due to phosphorus derived from phosphoprotein. To study this anomalous post-irradiational reaction, instead of performing in duplicate the primary digestion on the residue containing total nucleic acids at 18 hours to liberate the RNAP component from the DNAP, two separate primary analyses, one at 18 hours and the other at 6 or 12 hours were carried out on each half of the residue. This technic of varying the time of hydrolysis was instituted after analyses on Experiment No. 26 were completed, but was carried out on the 6 remaining experiments of Group III, as well as in No. 3 of Group I. The results in the latter experiment are difficult to interpret as little difference in values of DNAP and RNAP occurred with hydrolysis in the separate halves of the residue. In Group III, the time at which the anomalous reaction related to increase of RNAP with associated decrease in DNAP occurred, varied between 30 and 90 minutes in different experiments. In No. 29, it was first noted at 30 minutes, in Nos. 30, 31 and 32 at 60 minutes and in Nos. 26, 27 and 28 at 90 minutes. Lessening the time of primary hydrolysis on half of the residue below 18 hours, resulted in a lowering of the RNAP and an increase in the DNAP values as compared to figures obtained at 18 hours hydrolysis.

In Experiment No. 26, the abnormal rise in cellular RNAP associated

with a fall in DNAP beginning at 90 minutes when plotted on a correlation graph in Fig. 6, appeared as an aberrant curve extending upward and to the left. Data from Experiment No. 26 was interesting because of the high initial cellular DNAP value, namely 1.50×10^{-9} mg and the height of the

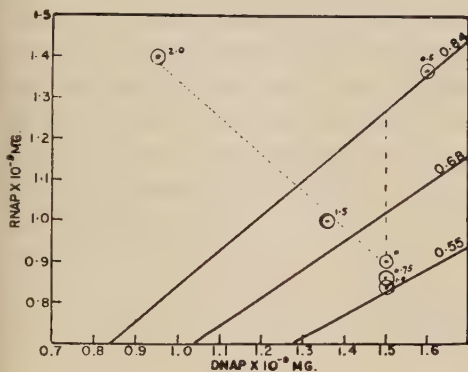


Fig. 6.

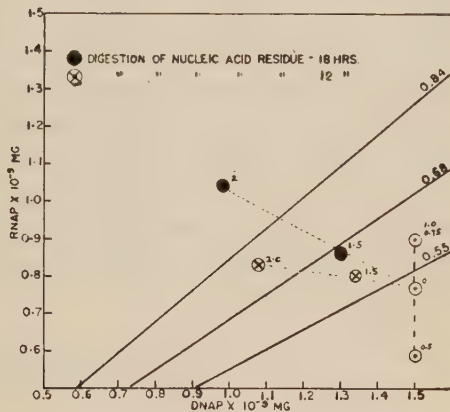
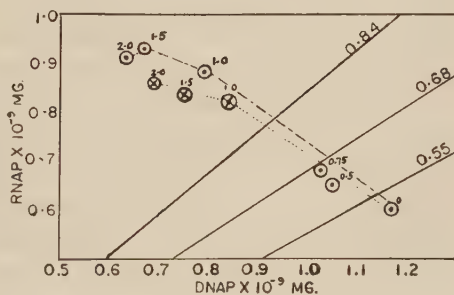


Fig. 7.

6. Changes in cellular DNAP and RNAP of a suspension of cell of mouse mammary carcinoma following exposure to X-ray; dosage 100 r. The point at 4 hours is higher than expected and is probably due to instability of DNAP in the nucleic acid residue during primary digestion. Data from Expt. No. 26 in Table I, group III.

7. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 100 r. Data from Expt. No. 28 in Group III.

8. Changes in cellular DNAP and RNAP of a suspension of cells of mouse mammary carcinoma following exposure to X-ray; dosage 100 r. Points at 1 and 2 hours indicate definite instability of the DNA in the residue, when this was subjected to alkaline digestion. Data from Expt. No. 29 in Group III.



rise in RNAP. Similar aberrant curves, but in pairs (with dual times for hydrolysis) which diverged terminally, were developed in the other experiments of Group III following brief X-radiation of 100 r. Only two of these, namely Fig. 7, plotted from data of Experiment No. 28, and Fig. 8, plotted from data of Experiment No. 29, are reproduced. In Fig. 7, a considerable

divergence developed between the pair of abnormal lines as they extended upward and to the left. This divergence was interpreted as indicating the presence of a greater quantity of DNAP, namely 1.50×10^{-9} mg per cell, to undergo partial dissolution following irradiation. In Fig. 8, in which the anomalous reaction occurred on the first analysis (30 minutes after irradiation) the paired lines on extension upward and to the left reach a point at which they turn and move downward to the left. This latter movement downward and to the left, simulated the reaction found generally in nucleic acids of cells undergoing retrograde changes. One other observation made in these experiments in Group III is that the partial dissolution set up by X-radiation is graphically shown by lines extending upward and to the left from the initial values of the nucleic acids, rather than from the lowest value to which the nucleic acids may decrease.

DISCUSSION

Our experiments relating to changes in nucleic acid following X-radiation gave rise to more intricate findings than those generally encountered by investigators engaged in the enumeration of percentages of temporary suppressions and recoveries in mitoses resulting from X-radiation. In only a few instances did our curves simulate the simple curves recorded by Strangeways (9, 10) and later workers (2), of transient decrease and gradual recovery in the number of mitotic figures induced in cell cultures by low X-ray dosage. That exposure of cells to X-ray of low dosage may stimulate anabolic activities was recently shown by Daniel and Park (1) who produced reproduction in *Paramecium* above that of the controls with continuous X-radiation of 4 to 8 r. per hour. Despite the lability and complexity of our cellular material, results thus far obtained warrant, we believe, certain conclusions. The first is that we are not dealing with a temporary restraint to the vital processes underlying mitosis followed by gradual release of these processes from the restraint. An actual synthesis of nucleic acid stimulated by irradiation appears to be taking place from precursors existing in the tumor cells suspended in the Ringer's solution. The X-ray dosage is a determining factor activating this synthesis and the limits of this factor are narrow. A dosage of 150 r. is beyond the physiological stimulus for synthesis; 100 r. and possibly 75 r. are sufficient to induce synthesis of nucleic acid if adequate chemical precursors are present in the cell, but the dosage of 100 r. produces, in addition to formation of nucleic acids, a certain amount of chemical instability in

the synthesized material. The duration of the anabolic activities in isolated cells is not beyond 4 hours, which agrees with the duration of recovery from inhibited mitoses reported by Strangeways *et al.* (9, 10). Necessary requisites for synthesis in the cells of the mammary tumor used, was cellular DNAP above that commonly possessed by the normal cell, and a RNAP/DNAP ratio above 0.55. A second conclusion growing out of our findings, is that the quantitative and qualitative variations in the two nucleic acids obtained experimentally both by temperature and irradiation, give a graphic pattern by which nucleic acids are decreased. X-radiation, in addition, may give a pattern of increase within the cell. Graphically, increase and decrease follow the same routes but they are in reverse. The graphical evidence presented indicates that under certain conditions RNA activities may occur in two different ways; one, taking place along the lines of regression and associated with DNA, and the other occurring in a perpendicular migration between the lines of regression along points with fixed values of DNA, but not associated with changes in the latter nucleic acid.

SUMMARY

1. X-radiation of 150 roentgens caused a continuous decrease in the nucleic acid content of cells of a mouse mammary carcinoma uniformly suspended in Ringer's solution. A dosage of 100 r. produced a variable increase in cellular nucleic acids, which appeared to be correlated with high RNAP/DNAP ratios. We believe that this dosage of X-ray caused some increased susceptibility of DNA to alkaline hydrolysis, as shown by the Schmidt-Thannhauser technic (6), resulting in an abnormal increase in RNAP from 30 to 90 minutes after exposure. A dosage of 75 r. produced changes in cellular nucleic acid difficult to interpret.

2. The increase in nucleic acids following X-radiation of 100 r. was interpreted as a synthesis from precursors existing in the cell rather than a recovery from a temporary inhibition in their development.

3. Increase and decrease in RNAP following X-radiation are shown graphically in the accompanying figures in two ways; one, associated with DNAP and taking place up and down along the lines of regression, and the other, not associated with DNAP and occurring in a perpendicular line up and down between lines of regression at points with fixed values of DNAP. In our experiments recorded here, these values of cellular DNAP are 1.00, 1.20 and 1.50×10^{-9} mg.

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OBSERVATIONS ON THE PHYSICO-CHEMICAL PROPERTIES OF ISOLATED NUCLEI¹

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PRACTICALLY nothing is known of the mechanisms by which nuclear substances are transferred to the cytoplasm where they determine, directly or indirectly, cellular physiology and structuration.

When, during mitosis, the nuclear envelope, or karyotheca, breaks down, the non-structured content of the nucleus is free to mix with the cytoplasm. It may be doubted, however, that it is only during the short and irregularly occurring periods of mitosis, during which the chromonemata appear as heavily insulated units, that gene products leave the nucleus. Evidence rather suggests that gene products affect cytoplasmic manifestations continuously while the nuclear envelope remains intact (18). In some instances it could actually be observed that nuclear products—not necessarily of a specific genic nature—are poured into the cytoplasm during non-mitotic stages. They may be emitted in the form of vacuoles, granules, or histochemically characterized substances, as for example, in the maturing oöcytes of *Limnaea* (17).

In most instances, the transfer of nuclear materials into the cytoplasm of a resting cell has eluded optical detection. Yet one must assume that it occurs in every cell on a molecular level. It is also necessary to assume that raw materials pass from the cytoplasm into the nucleus permitting the nuclear components, not only to maintain metabolism, but, in addition, permitting the synthesis of gene replicas and gene products. Since it is difficult to imagine that compounds short of a highly complex molecular order represent the specific gene messages, it appears desirable to investigate whether or not the nuclear envelope is actually permeable to macromolecules.

¹ This paper is dedicated to Professor F. Baltzer, Universität Bern, on the occasion of his 70th birthday, as a token of my admiration for him as a scientist, teacher and grand person altogether.

The work was done under a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

The impetus to approach this problem experimentally I owe to Dr. A. L. Dounce of this university. He also provided me with the various immersion media which he had used previously in his extensive studies on isolated mammalian nuclei (7, 8, 9). It was one of our objects to examine the effects of these media upon freshly isolated single nuclei as revealed by continuous microscopic observation.

In order to study the permeability of the nuclear envelope to high weight molecules, solutions of hemoglobin from beef were chosen. It was thought that a diffusion of this colored protein into the nucleus would be readily detectable simply by observing the fresh nuclei at low magnification with the microscope.

EXPERIMENTAL RESULTS

The following experiments were carried out on the isolated nuclei, or germinal vesicles, from mature oöcytes of *Rana pipiens*. Because of their enormous size (approximately 0.5 mm in diameter) and because of the ease with which they can be isolated with the aid of glass dissecting-needles, the germinal vesicles of amphibian eggs have been praised since the times of O. Hertwig as a most suitable material for structural and physiological studies. Partly in confirmation of the data of Duryee (10, 11, 12) and Callan and Tomlin (6) on various amphibian species, the conditions in the present material were found to be as follows.

In a favorable immersion fluid, the freshly isolated germinal vesicle of *Rana pipiens* oöcytes represents a transparent, more or less spherical body, the surface of which projects into irregularly shaped bulges. The interior contains a gelated core which can be dragged out of the nucleus by means of glass needles and which forms the embedding material for the chromosomes and for a great and varying number of "nucleoli" of different sizes. The normally gelated core is separated from the karyotheca by a colloidal fluid which, when released by puncturing the nucleus, mixes freely with calcium-free aqueous media.

The properties of the nuclear envelope, as tested by mechanical and chemical devices, differ markedly from those of the peripheral plasma membrane of embryonic amphibian cells. When the envelope of an isolated germinal vesicle is torn mechanically, the hole is not sealed by convergent movements of the remaining surface layer, as occurs in punctured and healing cell surfaces (14). Instead, the torn karyotheca retains sharp edges, even in media which may be considered as physiologically favorable; no marked

amount of nuclear content is squeezed out because no contraction of the envelope takes place. Probing with glass needles shows that this membrane, in contrast to the plasma membrane, represents a tough, though pliable structure lacking elastic tension. Yet the isolated nucleus can be made to expand and contract reversibly under the influence of turgor changes of the karyoplasm.

When germ vesicles are kept for about a day in solutions which have been used by Dounce and others as suspension media for isolated liver nuclei (0.127 to 0.25 M sucrose plus 0.0018 M CaCl_2), or in a physiological salt solution isotonic for embryonic amphibian cells (13, 14), the nuclear envelope evidently undergoes an irreversible change in physicochemical properties. Its normally flabby, pliable state changes into that of a paper-like, rigid consistency. Since the procedures involved in isolating the nuclei from adult tissues require a good day's work, it is likely that these nuclei too, before they are ready for biochemical investigation, have undergone a comparable change.

The karyotheca furthermore differs from the cell membrane in its chemical reactions. Cobra venom, or high concentration of sodium oxalate which readily dissolve the plasma membrane of embryonic amphibian cells (14), had no visible effect upon the nuclear envelope. Trypsin, on the other hand, which has no, or hardly any, effect upon living amphibian cells (19), dissolved at least most of the karyotheca. Exposure of freshly isolated nuclei to KOH of high concentration (pH above 12) produced only a partial solution of the envelope while the remainder expanded into an extremely thin continuous film. A similar transformation resulted from the application of HCl (pH below 4). In the "denatured" state, however, the envelope remained apparently unaffected by very high or low pH. Ethyl alcohol or xylene produced shrinkage and stiffening but no dispersion of the karyotheca.

The karyotheca is sufficiently thick to become visibly stained with basic dyes. Toluidine blue stained it violet. Millon's reagent produced a distinct coloration of the envelope as well as of the inner jelly of the nucleus. When subjected to the forces of surface tension at the air-liquid interface, fresh, in contrast to physicochemically altered nuclei, were torn apart into fragments.

Evidently the karyotheca is considerably thicker and contains comparatively more protein and less lipids than does the outer plasma membrane. According to the optical-polarization and electron-microscopic studies of Callan and Tomlin (6), the karyotheca of amphibian oöcyte nuclei consists of two layers: an outer lipo-protein sheet which in a desiccated and there-

fore highly altered state is some 300 Å in thickness and exhibits regularly arranged pores with diameters of approximately 400 Å, and an inner, homogeneous-appearing sheet about 150 Å in thickness which is thought to determine the permeability of the nuclear envelope. Similar structures have been found in the envelope of amoeba nuclei (2). A lipo-protein composition has been assumed to characterize the karyotheca of the nuclei in a variety of other cells (reviewed by Baud, (3), Anderson, (1)).

On the basis of these data it is difficult to visualize how large molecules can pass through the apparently densely knit inner nuclear layer (and through the outer layer which in a living, hydrated state is likely to have its "pores" covered by some film, possibly of phospholipids¹), simply by way of diffusion. Nevertheless, such a passage evidently occurs.

Before discussing some aspects of permeability of the nuclear envelope, I should like to deal briefly with *methodological problems* which are of utmost importance for the evaluation of the data obtained from the biochemical study of isolated nuclei.

Attempts to isolate germinal vesicles free of adhering cytoplasmic components and at the same time without loss of their genuine structure and composition meet with the same difficulties which have become a familiar dilemma and the source of controversies in the work on isolated nuclei from adult mammalian tissues. In the present case, the complications arising from the use of a Waring blender or other mechanical devices to free the nuclei from the cytoplasm do not exist. But here, too, it is a matter of choosing adequate immersion fluids in order to obtain "clean" nuclei. When the dissection of the oöcyte nucleus is carried out in the calcium containing salt solution devised for culturing whole amphibian cells (13), considerable amounts of cytoplasm remain firmly stuck to the nuclear surface. Such preparations appear to be unsuited for the biochemical analysis of purely nuclear components. The enzyme determinations which Brachet (4) made on such contaminated germ vesicles must, therefore, be interpreted with caution.

Clean germ vesicles can be obtained in media lacking calcium. In our experiments distilled water, or saline, or sucrose solutions with a pH range of between 6 and 8, combined with mechanical rinsings, produced nuclei

¹ A closely packed layer of small lipo-protein vacuoles at the cell surface may simulate a sieve-like sheet; upon dehydration this "network" partly disintegrates into granules (16). According to the principles of coacervation, a homogeneous film of hydrated impure phospholipids tends to form a similar "fenestrated" sheet with numerous vacuoles or "pores" when it is undergoing desiccation (15). These and other observations appear to be pertinent if one attempts to relate the data on the porous structures of desiccated nuclear membranes to normal conditions.

free of visible cytoplasmic contamination. However, in these media the nuclei swell considerably. The nucleoli are dissolved and it is likely that, also, the nuclear membrane and the karyoplasm suffer degradation and loss of substance, probably even of certain proteins. Thus, biochemical studies of the composition of "clean" nuclei are liable to reveal less substances than are present in the experimentally undisturbed nuclei. At present there seems to be no way of overcoming this dilemma.

On the other hand, isolated nuclei may also readily take up new compounds from the suspension media. This becomes apparent from the following studies on the specific gravity and the *permeability* of the germ vesicles. When isolated by micro-dissection in distilled water or in one per cent gum arabic, or in a physiological salt solution isotonic for whole amphibian cells, the nucleus remains at the bottom of the operation dish. But it will float to the surface when transferred immediately to the following aqueous solutions: 5 per cent gum arabic, 70 per cent ethylene glycol, or sucrose solutions above 0.167 M.

Both gum arabic and sucrose can readily diffuse into the nucleus because in these new media the floating nucleus will sink to the bottom within about two minutes. When returned to any one of the initial immersion fluids and then transferred once more to sucrose or gum solutions of a higher concentration, the nucleus will repeat this performance.

A useful criterion for the study of some diffusion phenomena is the behavior of the *nucleoli*. Germ vesicles which are isolated in distilled water or in sucrose solutions lacking calcium lose their nucleoli in less than a minute. When sodium oxalate is added to the calcium containing physiological suspension medium, the nucleoli likewise disappear. This occurs stepwise; at first adjacent nucleoli become confluent; then they dissolve entirely. It appears, therefore, that calcium ions are essentially involved in preserving the integrity of the nucleoli. The rapidity with which the nucleoli respond to these treatments indicate an unimpeded passage of water and electrolytes through the nuclear envelope.

According to a brief report of Waddington (20) concerning the studies of Callan, the lipo-protein envelope of the isolated nuclei of amphibian oöcytes is permeable to molecules of the size of sucrose, dextrin or depolymerized nucleic acids, but not to substances such as plasma albumin or glycogen. We have obtained data which indicate that *hemoglobin*, a moderately large protein molecule, can readily pass through the nuclear envelope. A corresponding claim has been made by Anderson (1) with regards to liver nuclei although details in support of this claim have not been given.

The penetration of beef hemoglobin into the oöcyte nuclei could be observed in the following immersion fluids: distilled water, gum arabic, sucrose or balanced salt solutions of different concentrations. In all of these instances, the red color of hemoglobin could be detected inside the nucleus within periods of between 10 and 30 minutes, these variations being dependent, not upon the kind of immersion fluid, but upon the concentration of the hemoglobin solutions used. When returned to fresh media, the nuclei lost the hemoglobin as fast as they had gained it. This rapid turn-over occurred in nuclei immediately after isolation as well as in stale nuclei which were obviously changed in regard to physicochemical properties. The passage of hemoglobin in either direction could be obtained repeatedly.

Evidently, the transport of hemoglobin across the nuclear envelope is simply due to diffusion and not to specific properties of a semipermeable membrane. It appears, therefore, that the nuclear envelope of the frog oöcyte offers no obstacle to the free passage of medium-size protein molecules. The preceding data on sucrose and gum arabic likewise indicate that the nuclear envelope does not act like a semipermeable membrane comparable to the plasma membrane of the cell surface. This conclusion which has been drawn previously by other authors (8, 1) is difficult to connect with the data on the composition and structure of these two kinds of membranes. According to chemical and other studies both the nuclear and the cellular membrane consist predominantly of proteins and lipids. The nuclear membrane is evidently much thicker than the cell membrane. Yet the former seems to be far more permeable than the latter. It appears that electron microscopic studies cannot help very much in solving this riddle.

One might expect that the freshly isolated germ vesicle, when kept in an artificial medium found to be favorable for the culture of whole cells, would retain its genuine properties during the short period of time which is involved in the studies here reported. Nevertheless, we hesitate to attempt to transfer these data to nuclei as they exist within normal cells. It is true that the experimental data concerning the free passage of macromolecules across the karyotheca support the hypothesis of free diffusion presented above. On the other hand these data do not prove that such a transfer occurs in living cells. Actually these studies should be carried out on nuclei within their normal cytoplasmic environment. Such experiments are in progress.

In this connection reference should be made to the recent paper of Briggs and King (5). These workers found that the composition of the suspension medium is of crucial importance for the preservation of the genuine properties of isolated nuclei of frog embryos. They observed that the "Holtfreter solu-

tion" referred to above was relatively favorable, whereas a sojourn of probably not more than one or a few minutes in cytoplasmic homogenates, or in the calcium-free standard solution of Duryee, damaged the nuclei to such an extent that they failed to undergo mitosis when they were subsequently injected into an enucleated frog egg. It is these subtle yet physiologically significant changes of living matter which are of principal interest to the biologist, but which are unfortunately quite refractive to a physico-chemical characterization.

Independently of the application of our data to normal conditions, they are valuable in so far as they throw doubt upon the conclusiveness of the results obtained by various authors with regards to the presence or absence of hemoglobin and other proteins and enzymes in nuclei isolated in aqueous media. If as in the present experiments, a protein can pass across the envelope of a freshly isolated nucleus within a few minutes, then one is at liberty to assume that in the drawn-out process of isolating nuclei from adult tissues there is ample time for the diffusion of proteins from the cellular homogenate into the nuclei, and vice versa. Anderson (1) reports that nucleases and bovine serum albumin can rapidly penetrate into freshly isolated unfixed rat liver nuclei. Hence it is an open question as to what extent the data concerning the enzyme content of "clean" nuclei isolated in aqueous media are valid with regards to living non-isolated nuclei.

SUMMARY

Beef hemoglobin, sucrose, and gum arabic readily pass through the nuclear envelope of the germinal vesicles of the frog oöcyte isolated in different media. The implications of these and other observations on the physico-chemical properties of isolated nuclei are discussed with reference to the studies on the chemical composition of isolated nuclei from adult tissues.

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ZENTRIFUGIERVERSUCHE ÜBER DEN EINFLUSS BEFRUCHTUNGSFÖRDERNDER UND HEMMENDER SUBSTANZEN AUF DEN ZUSTAND DER OBERFLÄCHE DES SEEIGELEIS

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Es konnte gezeigt werden, dass die Befruchtungsfähigkeit der Eier von *Psammechinus miliaris* von jahreszeitlichen Bedingungen und dem Allgemeinzustand der Eier abhängig ist (4, 5). In früheren Untersuchungen ist nachgewiesen worden, dass eine Reihe verschiedener Substanzen einen wesentlichen Einfluss auf die Befruchtungsfähigkeit ausüben (8, 10, 11, 17, 18). Bei Eiern die sich im Zustand der zytoplasmatischen Unreife befinden und die darum nur schlecht befruchtet werden können, liess sich z. B. durch Behandlung mit Aminosäuren (17, 18, 19) sowie Adenosintriphosphat (6) oder Natriumperjodat (8) eine wesentliche Verbesserung der Befruchtung sowohl in quantitativer als auch in qualitativer Hinsicht erreichen. In ähnlicher Weise wirkten bestimmte Globinpräparate oder Seidenpepton (19) sowie auch aus Eiern oder Sperma gewonnene Extrakte (2). Auf die speziellen chemischen Wirkungen, die von diesen Stoffen ausgelöst werden können, soll hier nicht eingegangen werden. Der Zweck der vorliegenden Arbeit ist, zu untersuchen welchen gleichartigen Einfluss die erwähnten Stoffe auf den Zustand der Oberfläche des Seeigeleis haben, da es sich gezeigt hat, dass dieses von wesentlicher Bedeutung für die Befruchtungsfähigkeit der Eier ist (19). Die erwähnten Substanzen verursachen eine mehr oder minder starke Herabsetzung der Rigidität der Plasmamembran der unbefruchteten Eier. Diese Wirkung liess sich mit Hilfe des Zentrifugenmikroskops nachweisen (3, 7, 19). Für die vorliegenden Untersuchungen war es von Interesse zu ermitteln, welchen Einfluss die Konzentration verschieden wirksamer Verbindungen auf die Streckung der Eier während der Zentrifugierung ausübt sowie auch weitere Substanzen mit ähnlicher Wirkung zu prüfen.

In früheren Untersuchungen hat sich gezeigt, dass Substanzen mit befruchtungshemmender Wirkung gleichfalls die Rigidität der Plasmamem-

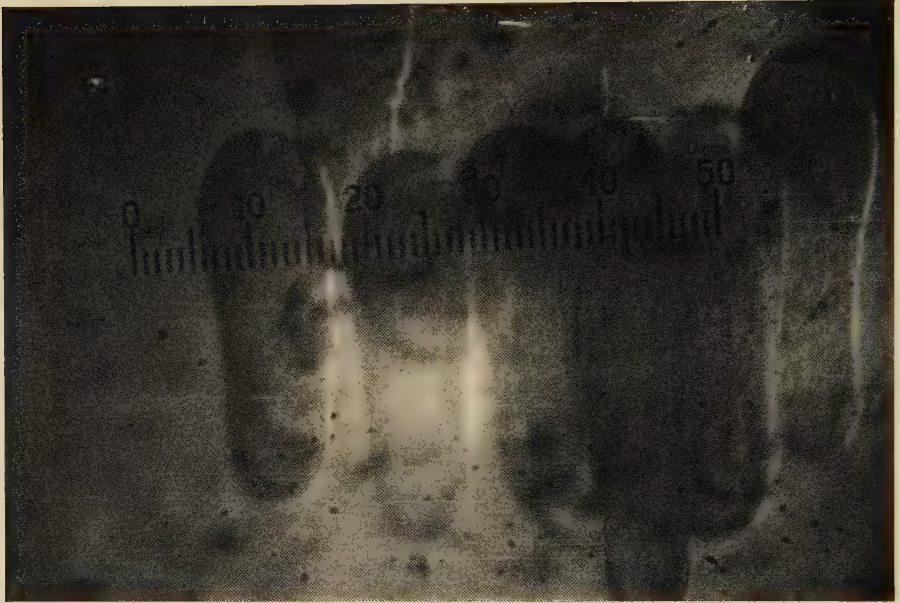


Abb. 1. Streckung von Eiern die mit 0,001 M Thioglykolat behandelt wurden im Zentrifugenmikroskop nach 7 Minuten.

bran beeinflussen (3, 19). In diesem Fall wurde die Rigidität der Plasmamembran der unbefruchteten Eier wesentlich erhöht. Die Eier zeigten eine geringere Streckung während des Zentrifugierens, als unbehandelte Kontrollen. Der wirksame Faktor dieser aus *Fucus vesiculosus* gewonnenen Präparate, der die Befruchtungsfähigkeit hemmt und die Rigidität im Kortex herabsetzt, ist als „Hemmungsfaktor F“ bezeichnet worden (3). Im Zusammenhang mit den ersterwähnten Befunden war es von Interesse, weitere solcher Präparate zu untersuchen und die Resultate mit den übrigen Versuchen zu vergleichen.

MATERIAL UND METHODE

Die Versuche wurden während des Sommers an der zoologischen Station Kristineberg an der schwedischen Westküste durchgeführt. Für alle Zentrifugerversuche wurden die Eier von *Psammechinus miliaris* benutzt, die in einem Seewasser von ca. 25 pro mille gehalten wurden. Diese Konzentration entspricht ungefähr dem normalen Medium der litoralen Form dieser Art. Die Gallerthülle der Eier ist in allen Versuchen durch kurzfristiges Waschen in schwach angesäuertem Seewasser (pH 4,5) entfernt worden. Die Eier wurden danach für 30 Minuten mit den

folgenden im Text erwähnten Verbindungen behandelt und anschliessend in eine Mikroküvette übertragen, um dort für die Dauer von 8 Minuten in einem Zentrifugenmikroskop (Fabrikat Struers, Kopenhagen) mit einer konstanten Zentrifugalkraft von 4350 G. zentrifugiert zu werden. In der Mikroküvette waren die Eier zwischen Seewasser und einer isotonischen Rohrzuckerlösung suspendiert. Während des Zentrifugierens strecken sich die Eier zwischen der Grenzschicht der beiden Lösungen. Diese Streckung wurde mit einem Messokular bestimmt und mit der unbehandelten Kontrollen verglichen. Während des Zentrifugierens befanden sich durchschnittlich ca. 10–15 Eier im Beobachtungsfeld (siehe Abb. 1). Von diesen wurde während des Zentrifugierens in gleichmässigen Abständen von 1 Minute die Streckung gemessen. Für die Bestimmung der Streckung ist der Mittelwert der während dieser Zeit gemessenen Werte berechnet worden. Die in der Fig. 1. dargestellten % Zahlen geben diese Mittelwerte der Streckung während des Zeitraums von 8 Minuten in Abständen von 1 Minute wieder.

Bei den in der Tab. I angegebenen %-Zahlen handelt es sich um die Mittelwerte aus je 2–4 Versuchen.

Die Eier befanden sich vor dem Zentrifugieren in Schalen mit Seewasser von ca. 15–16°. Während des Zentrifugierens betrug die Raumtemperatur 17–18°. Es wurde darauf geachtet, dass die Zentrifuge während des Versuchs die gleiche Temperatur behielt und dass sie sich nicht infolge zu häufiger Benutzung erwärmte, da die Streckung von der Temperatur beeinflusst wird und bei einer Temperatursteigerung zunimmt.

Einige der oben erwähnten Aminosäuren sind in Konzentrationen von 0,05 M bereits in früheren Versuchen geprüft worden (19), dabei zeigte sich in fast allen Versuchen eine etwas stärkere Streckung, sowohl der mit den einzelnen Verbindungen behandelten Eier als auch der unbehandelten Kontrollen, da in dieser Versuchsserie bei einer Temperatur von 22–24° gearbeitet wurde. In den vorliegenden Versuchen liegen die Werte für die Streckung der Eier etwas tiefer da die Temperatur 17–18° betrug.

Um die Rigidität des Zytoplasmas durch die Messung der Schichtung der Plasmakomponenten zu bestimmen, wurden die Eier unter Anwendung einer konstanten Zentrifugalkraft von 4460 G. für die Dauer von 10 Minuten in einer gewöhnlichen Zentrifuge in Seewasser mit einem Zusatz von 2 % Dextran zentrifugiert. Die Schichtung des Zytoplasmas der mit den einzelnen Substanzen behandelten Eier wurde mit der einer unbehandelten Kontrolle verglichen (nähere Angaben über Methodik siehe (7)).

Die in den Versuchen verwendeten Präparate Sup C₅II, Sup C₄I und E₆I sind von Dr. Vasseur vom hiesigen Institut hergestellt worden. Sie wurden aus den Extrakten der Alge *Fucus vesiculosus* gewonnen, die reich an Polysacchariden waren. Diese Extrakte enthalten das saure Polysaccharid Fucoidin. Nach augenblicklich noch andauernden Versuchen ist der wirksame Faktor der Präparate nicht das reine Fucoidin sondern eine andere Substanz, die wahrscheinlich auch als Polysaccharid-Komponente bestimmt werden kann. In früheren Untersuchungen ist dieser Bestandteil, der die Rigidität im Kortex erhöht und die Befruchtungsfähigkeit hemmt, als „Hemmungsfaktor F“ bezeichnet worden (3). In den in englischer Sprache abgefassten Mitteilungen aus diesem Institut wird der Faktor abgekürzt als FeInh (Fu) bezeichnet, d. h. der befruchtungshemmende Faktor von *Fucus* (16).

Die von Dr. Eskil Hultin für die vorliegenden Versuche zur Verfügung gestellten Spermaextracte sind auf folgende Weise gewonnen worden.

Präparat Br 7 Sup 1:47 Ml. frisches, flüssiges Sperma von *Brissopsis lyrifera* wurden durch ein Baumwollfilter filtriert und für 5 Min. mit 8000/Umdr. in der Min. zentrifugiert. Die über dem Sediment (von 4,1 Gr.) stehende klare Flüssigkeit wurde in einer Verdünnung von 1:10 für die vorliegenden Versuche verwendet. Präparat Ec 8 Dial. Sup 1:14 Ml. frisches, flüssiges Sperma von *Echinocardium cordatum* wurden wie oben filtriert und zentrifugiert. Die über dem Sediment (von 2 Gr.) stehende klare Flüssigkeit wurde über Nacht gegen 1 Ltr. Seewasser dialysiert. Nach dem Dialysieren wurde die klare Flüssigkeit wie oben 1:10 mit Seewasser verdünnt und für die Versuche verwendet.

ERGEBNISSE

Beim Zentrifugieren normaler, unbefruchteter Eier erhält man im Verlauf von 7–8 Minuten eine Streckung von ca 55–65 %. Fig. 1 zeigt das Ergebnis von 10 Versuchen. Die in den Versuchen erhaltenen Mittelwerte sind im Zeitabstand von je einer Minute eingetragen worden. Die ausgezogene Linie entspricht den im Durchschnitt am häufigsten gemessenen Werten. Die Tabelle I zeigt die Streckung der für die Dauer von 8 Minuten zentrifugierten Eier für verschiedene Konzentrationen von Aminosäuren im Vergleich mit unbehandelten Eiern des gleichen Materials. Eine Erhöhung der Konzentration bewirkte eine stärkere Streckung der Eier. Bei stark wirk-samen Verbindungen wie z. B. Thioglykolat treten diese Unterschiede deutlich hervor, wie aus dem Vergleich der in Tab. I dargestellten Werte für die verschiedenen Konzentrationen zu entnehmen ist. Die Abb. 1 zeigt die

TABELLE I

Konzentration in Mol.		Streckung in % nach 7 Min.	Kontrolle. Streckung in % nach 7 Min.
0,05	Histidin	115	65
0,025	»	80	65
0,05	Glykokol	105	55
0,025	»	85	55
0,1	Oxyprolin	100	55
0,05	»	85	65
0,1	Prolin	95	55
0,1	Serin	120	57
0,025	Glutathion	95	60
0,01	Thioglykolat	135	55
0,001	»	110	55
0,0001	»	75	55

starke Streckung der Eier, die mit 0.001 M Thioglykolat behandelt wurden, während des Zentrifugierens im Zentrifugenmikroskop. Glutathion bewirkte gleichfalls eine Verlängerung der Eier (Tab. I). In früheren Untersuchungen hat sich gezeigt (19), dass einige Aminosäuren wie z.B. Prolin, Oxyprolin und Serin bei einer Konzentration von 0,05 M keine wesentlichen oder nur sehr schwache Wirkungen zeigten. Erhöht man die Konzentration dieser

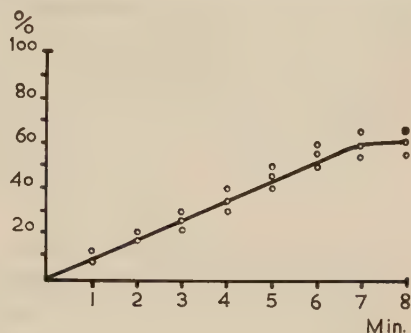


Fig. 1. Streckung unbehandelter unbefruchteter Eier von *Psammechinus miliaris* im Zentrifugenmikroskop. Die dargestellten Werte zeigen die Streckung in % über den Normaldurchmesser an.

Verbindungen auf 0,1 M, wie es in den vorliegenden Versuchen geschehen ist, so tritt eine deutliche Wirkung auf (siehe Tab. I). Die Wirkung von Spermextrakten geht aus Tab. II hervor. Tab. II zeigt gleichfalls das Ergebnis von Versuchen mit verschiedenen aus *Fucus vesiculosus* hergestellten Präparaten mit dem „Hemmungsfaktor F“. Die stärkste Wirkung hatte das Präparat Sup C₅II, das in 0,05 %iger Lösung die Streckung der Eier weitgehend hemmt. Präparat E₆I zeigte in gleicher Konzentration eine ähnliche und Sup C₄I eine etwas schwächere Wirkung.

Wie bereits in früheren Versuchen festgestellt, wird die Rigidität des Zytoplasmas durch eine Reihe von Aminosäuren mehr oder weniger herabgesetzt. Dieses kann durch Zentrifugieren der Eier in einer gewöhnlichen Zentrifuge nachgewiesen werden, wobei die Schichtung der Zytoplasma-

TABELLE II

Konzentration		Streckung in % nach 7 Min.	Kontrolle. Streckung in % nach 7 Min.
1:10 mit Seewasser verd.	Sperm. Extr. Br. 7 Sup. 1	130	60
1:10 mit Seewasser verd.	Sperm. Extr. Ec. 8 Dial. Sup. 1	95	55
0,05 %	Sup. C ₅ II	10	60
0,05 %	E ₆ I	15	60
0,05 %	Sup. C ₄ I	25	60

komponenten unter konstanten Bedingungen bestimmt wird. Es handelt sich dabei um verhältnismässig schwache Effekte. Eine geringe Herabsetzung der Rigidität des Zytoplasmas hat keinen wesentlichen Einfluss auf die Streckung der Eier beim Zentrifugieren, da in diesem Fall der Zustand der Oberfläche für das Ausmass der Streckung bestimmend ist. Eine stärkere Wirkung auf die Rigidität des Zytoplasmas hatte der Spermextract Br. 7 Sup. 1. Die damit behandelten Eier zeigten nach dem Zentrifugieren eine stärker ausgeprägte Schichtung als unbehandelte Kontrollen. Bei den mit dem „Hemmungsfaktor F“ behandelten Eiern war keine wesentliche Änderung der Zytoplasmarrigidität feststellbar.

BESPRECHUNG DER ERGEBNISSE

In früheren Versuchen konnte nachgewiesen werden, dass der Zustand der Unreife der Eier nach Abschluss der Kernreifung im Hinblick auf das Zytoplasma noch anhalten kann (5). Unreife Eier lassen sich schlecht befruchten. Der Zustand der Unreife ist auch mit Hilfe der Hypertoniemethode nachweisbar, wobei solche Eier durch eine länger andauernde Faltigkeit der Oberfläche gekennzeichnet sind, während bei reifen Eiern dieser Zustand schneller verschwindet (5, 9). Nach Behandlung mit Substanzen die eine Herabsetzung der Rigidität der Oberfläche bewirken, zeigt sich im Hypertonieversuch, dass die Faltigkeit der Oberfläche bei diesen Eiern eher verschwindet als bei unbehandelten Kontrollen. Wicklund (19) konnte mit dieser Methode in Hypertonieversuchen die verschiedenartige Wirkung von Aminosäuren auf die Oberfläche der Eier nachweisen, indem sie die Zeitdauer bestimmte, die bis zum Verschwinden der Faltigkeit vergeht. Die mit dieser Methode gewonnenen Ergebnisse sind in Übereinstimmung mit den Befunden der vorliegenden Versuche, bei denen die Veränderungen der Oberfläche mit Hilfe der Zentrifugiermethode nachgewiesen wurden.

Auf der anderen Seite lässt sich zeigen, dass Substanzen welche die Rigidität der Oberfläche herabsetzen, meist auch die Befruchtungsfähigkeit der Eier verbessern (19). Häufig haben diese Stoffe auch gleichzeitig eine aktivierende Wirkung, so dass sie mehr oder weniger starke Vorgänge in dieser Richtung oder auch teilweise parthenogenetische Entwicklungen, z.B. unvollkommene Zellteilungen der Eier, verursachen können. Diese Wirkungen sind nicht nur von der Art, sondern auch von der Konzentration und der Einwirkungsdauer der betreffenden Substanz abhängig. Für eine Reihe der hier untersuchten Verbindungen konnte gezeigt werden, dass ihre Wirkung auf die Oberfläche der Eier von der Konzentration abhängig ist. Bei stärkerer

Konzentration nahm die Verlängerung der Eier im Zentrifugerversuch zu. Teilweise war die Wirkung auch erst von einer bestimmten Konzentration ab sichtbar. Der Einfluss der Konzentration war auch in den Hypertonieversuchen von Wicklund (17) nachweisbar.

Es hat sich gezeigt, dass eine Reihe der hier untersuchten Verbindungen auch eine Wirkung auf die Lebensdauer und das Befruchtungsvermögen der Spermien ausüben (13, 14, 15, 18): In der vorliegenden Untersuchung ist aber nachgewiesen worden, dass der Zustand der Eioberfläche durch die hier untersuchten Verbindungen wesentlich beeinflusst werden kann. Dies spricht dafür, dass die Aminosäuren und die übrigen erwähnten Verbindungen eine Verbesserung der Befruchtung im Sinne einer künstlichen zytoplasmatischen Reifung der Eier herbeiführen, was nicht eine partielle Wirkung auf die Spermien ausschliesst.

Auf der anderen Seite lässt sich zeigen, dass auch der Zustand, den die Eier bei der Unreife aufweisen, durch eine Reihe von Verbindungen hervorgerufen werden kann. Es handelt sich in diesem Fall um Lösungen von Gallerthüllensubstanz (12) oder verschiedene Präparate von Heparin (1), die in bestimmten Konzentrationen befruchtungshindernd wirken oder eine unvollkommene Ausbildung der Befruchtungsmembran hervorrufen. In ähnlicher Weise wirkten die Blutgruppensubstanz H (1) (aus Schweine-Mucin gewonnen) und die in früheren Arbeiten (3, 16, 19) erwähnten Präparate mit dem „Hemmungsfaktor F“, die aus *Fucus vesiculosus* hergestellt wurden. Alle diese Substanzen sind ihrer chemischen Natur nach Polysaccharide oder sie enthalten solche als Hauptkomponente. In einem Teil der Versuche konnte der Nachweis erbracht werden, dass durch Zusatz entsprechender Mengen von Natriumperjodat die befruchtungshemmende Wirkung dieser Stoffe aufgehoben wird (1). In der vorliegenden Untersuchung ist gezeigt worden, dass die aus *Fucus vesiculosus* gewonnenen Präparate die Rigidität der Oberfläche der Eier erhöhen und somit den Widerstand gegen die Streckung beim Zentrifugieren erheblich verstärken. In Parallele dazu konnte Wicklund (17) zeigen, dass im Hypertonieversuch die Faltigkeit der Oberfläche der Eier, die mit diesen Substanzen behandelt wurden, erheblich länger anhält als bei Nichtbehandelten. Weiterhin ergab sich in Zentrifugerversuchen (3), dass Eier die vorher mit dem „Hemmungsfaktor F“ und dann mit Natriumperjodat behandelt wurden, wieder eine stärkere Verlängerung zeigten. Nach Wicklund (16) wurden eine Reihe befruchtungshindernder Substanzen in ihrer Wirkung durch Albumin sowie durch Fraktionen von Eihomogenaten aufgehoben. Die hierbei vorliegenden chemischen Wirkungen bedürfen noch der weiteren Aufklärung.

ZUSAMMENFASSUNG DER ERGEBNISSE

1. Die Wirkung einer Reihe von Substanzen und Präparate, sowie die verschiedener Konzentrationen von Aminosäuren, auf die Plasmamembran der unbefruchteten Eier von *Psammechinus miliaris*, wird mit Hilfe des Zentrifugenmikroskops untersucht. Die Streckung der Eier wurde unter Anwendung einer konstanten Zentrifugalkraft gemessen und mit der unbehandelten Kontrollen verglichen.

2. Die Behandlung mit verschiedenen Aminosäuren sowie mit Thio- glykolat, Glutathion oder Spermextrakten bewirkte eine mehr oder weniger starke Streckung der Eier im Zentrifugerversuch.

3. Aus *Fucus vesiculosus* gewonnene Präparate erhöhten den Widerstand der damit behandelten Eier gegen die Streckung beim Zentrifugieren.

4. Im Zusammenhang mit den Befunden dieser Arbeit wird gezeigt, dass Substanzen welche die Rigidität im Kortex herabsetzen, befruchtungsfördernd wirkten während Verbindungen, die den entgegengesetzten Vorgang verursachten, die Befruchtung hemmten.

Am Ende dieser Arbeit ist es mir eine angenehme Pflicht, Herrn Prof. John Runnström für die Anregung zu den vorliegenden Untersuchungen und seine Hilfe meinen allerherzlichsten Dank auszusprechen. Herrn Dr. Erik Vasseur und Dr. Eskil Hultin danke ich für die mir zur Verfügung gestellten, in der Arbeit erwähnten Präparate. Weiter danke ich Herrn Prof. Nils Holmgren und Herrn Dr. Gunnar Gustafson von der zoologischen Station Kristineberg für ihre freundliche Hilfe und die ständige Beschaffung des für die Untersuchungen benötigten Materials.

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ÜBER DEN CHEMISCH INDUZIERTEN CHROMATIN- AUSTRITT AUS PFLANZLICHEN ZELLKERNEN

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DIE Tatsache, dass Chromatin unter verschiedenen Bedingungen aus den Zellkernen tierischer Gewebe in das Cytoplasma austreten kann, ist schon lange bekannt. So konnten z.B. Lewis (11) und Ludford (12) beobachten, dass in Gewebekulturen die Kerne manchmal Ausstülpungen bilden, die später abgeschnürt werden, und schliesslich daraus frei im Cytoplasma liegende Chromatinkörper entstehen. Nach einer Arbeit von Roskin (15) finden sich in degenerierenden Tumorzellen häufig Chromatinansammlungen, wobei entweder „hyperchromatische“ Kerne entstehen, oder eine Ausstossung des Chromatins ins Plasma stattfindet. Es handelt sich hier also durchwegs um scheinbar spontan entstehende Effekte.

An tierischen Zellen können durch Einfluss verschiedener Mitosegifte — Colchicin, Hydrochinon etc. — ähnliche Effekte an Ruhekernen erzielt werden (4). Auf Zellkerne pflanzlicher Zellen haben diese Reagenzien allerdings keinen derartigen Einfluss (9). Andererseits sind in den letzten Jahren mehrfach Befunde bekannt geworden, nach denen verschiedene Chemikalien und auch pflanzliche Extrakte einen Austritt des Chromatins aus dem pflanzlichen Zellkern in das Cytoplasma bewirken können. So berichtete Levan (8, 9, 10) über die Fähigkeit von Lithiumnitrat, Kaliumcyanid, sowie Penicillin G, solche Effekte hervorzurufen. Eine gleichartige Wirkung von Herbiciden wurde ebenfalls beobachtet (3, 13). Bei der Untersuchung der mutachromosomatischen Wirkung wässriger Extrakte von Zwiebeln verschiedener *Allium*-Arten konnte in unserem Laboratorium festgestellt werden, dass ein konzentrierter Extrakt aus Zwiebeln von *Allium sativum* im Wurzelmeristem von *Allium sativum* und auch von *Allium cepa* einen gleichartigen Chromatinaustritt bewirken kann. Wir haben für diese Erscheinung den Namen „Chromatinolyse“ vorgeschlagen (6).

Es erschien uns von Interesse, diese Erscheinungen näher zu untersuchen. In der vorliegenden Arbeit haben wir nunmehr mit cytochemischen und anderen Methoden versucht, die von verschiedenen Chemikalien verursachten Effekte näher zu analysieren.

METHODIK

Zwiebeln von *Allium cepa* und *Allium sativum* wurden zum Antreiben der Wurzeln in Leitungswasser getaucht und die Wurzeln bis zu einer Länge von etwa 2 cm anwachsen gelassen. Zur Behandlung brachte man die Wurzeln je 4, 24 und 72 Stunden in die entsprechenden Lösungen ein. Vor dem Fixieren der Wurzeln wurde kurz mit Leitungswasser gewaschen.

Lösungen folgender Substanzen wurden untersucht: LiNO_3 in den Konzentrationen 1 M, $3 \cdot 10^{-1}$ M, $1 \cdot 10^{-1}$ M, $3 \cdot 10^{-2}$ M und $1 \cdot 10^{-2}$ M; 2,4-Dichlorphenoxyessigsäure (2,4-D) $2,5 \cdot 10^{-2}$ M, $1 \cdot 10^{-2}$ M, $1 \cdot 10^{-3}$ M, $1 \cdot 10^{-4}$ M; Penicillin G (Na-Salz) 20 und 10 MIU/100 ccm, sowie ein wässriger Extrakt aus Knoblauchzwiebeln, der mit Hilfe eines „Waring-Blendors“ aus 40 g Zwiebeln und 150 ccm Leitungswasser gewonnen und anschliessend filtriert wurde. Die Acidität aller Lösungen wurde — wenn nötig — auf pH 7 nachgestellt.

Feulgen-Reaktion: Nach dem Fixieren in La Cour's 2 BD Fixiergemisch wurden die Wurzeln nach der üblichen Methode zu Quetschpräparaten verarbeitet und in Kanadabalsam eingeschlossen. Ein Teil der Wurzeln wurde in Paraffin eingebettet, Mikrotomschnitte von 12 μ Dicke hergestellt und der Feulgenreaktion unterworfen. In einigen Fällen wurden die Präparate mit einer Lösung von Lichtgrün F. S. (Grübler) in 40 % Alkohol behandelt, um eine Gegenfärbung zu erzielen.

Sakaguchi-Reaktion: Die Wurzeln wurden nach der Vorschrift von Serra (5) in einem Essigsäure-Alkohol-Formaliningemisch fixiert und in Paraffin eingebettet. Längsschnitte von 12 μ Dicke wurden nach dem Deparaffinieren nach der beschriebenen Methode der Reaktion unterworfen und in Glycerin eingebettet (16, 17, 18).

Methylgrünfärbung: Es wurde mit absolutem Alkohol fixiert und die Wurzeln anschliessend in Paraffin eingebettet (Temperatur nicht über 50° C). Die gewässerten Schnitte wurden 5 Minuten lang mit 0,01 N HCl behandelt, in 0,2 M Azetatpuffer pH 4,2 gewaschen und über Nacht in einer 0,25 m Lösung von Methylgrün in Azetatpuffer bei 5° aufbewahrt. Anschliessend wusch man 2 Stunden lang mit 0,05 M Azetatpuffer unter mehrmaligem Wechsel der Lösung (7). Als Farbstoff diente Methylgrün H 172 „Bayer“ (Hollborn). Zur Entfernung von violetten Begleitfarbstoffen wurde die Farbstofflösung mehrmals mit Chloroform extrahiert.

Ein Teil der Präparate wurde nach vollzogener Methylgrünfärbung einer Gegenfärbung mit einem Gemisch aus gleichen Teilen einer Methylgrünlösung (0,25 m) und einer Pyroninlösung (0,1 m) unterzogen (2). Man liess das Farbstoffgemisch 30 Min. einwirken und differenzierte danach durch 1-stündiges Waschen mit 0,05 M Azetatpuffer.

Feulgen-Reaktion

Nach 4- und 24-stündiger Einwirkung einer 1 M Lithiumnitratlösung konnte in nahezu allen Zellen des Wurzelmeristems ein Chromatinaustritt beobachtet werden. Feine Fäden, welche sich endständig zu kleinen tropfenförmigen Gebilden erweiterten, erstreckten sich radiär von der Kernoberfläche ins Plasma der Zellen. An der Austrittsstelle der Fäden zeigt die Kern-

oberfläche stachelige Erhebungen. Die beobachteten Chromatintröpfchen im Plasma entstehen anscheinend durch Abreissen der Verbindungsfäden; grössere Protuberanzen wurden bei diesem Versuch nicht beobachtet. An mehreren Zellen fiel es auf, dass alle Chromatintröpfchen konzentrisch in einem bestimmten Abstand von der Kernoberfläche angesammelt sind. Der Raum zwischen der Kernoberfläche und den Chromatinpartikeln im Plasma ist von einer optisch homogen erscheinenden Substanz erfüllt, die selbst feulgen-negativ ist, sich aber infolge geringfügiger Brechungsunterschiede vom Plasma unterscheidet. Es scheint, dass nach dem Austritt der thymonucleinsäure-haltigen Partikeln noch weiteres, feulgen-negatives Material ins Plasma ausgetreten ist.

Der Austritt von schwach gefärbten Substanzen aus Zellkernen des Wurzelmeristems von *Allium cepa* nach Einwirken einer Penicillinlösung (20 MIU/100 ccm) wurde bereits von Levan und Tjio (10) beschrieben. Wir konnten bei unseren Untersuchungen diese Erscheinungen sehr oft beobachten; es handelt sich in unserem Falle immer um feulgen-negatives Material, das bei normaler mikroskopischer Betrachtung nur schwer sichtbar ist. Werden feulgen-gefärbte Präparate dieser Art mit dem Phasenkontrastmikroskop betrachtet, erscheint das genannte Material dunkler als das umgebende Plasma. Diese Gebilde erreichen oft beträchtliche Grösse und sind auch an Kernen ohne Protuberanzen erkennbar (Abb. 14).

Nach 4-stündiger Behandlung mit einer 1 M Lösung zeigen Mitosestadien eine starke Verklebung der Chromosomen. In den Ruhekernen finden tiefgreifende Veränderungen statt; das Innere ist von grossen Vakuolen erfüllt, deren Inhalt feulgen-negativ ist.

Nach 24-stündiger Einwirkung einer $3 \cdot 10^{-2}$ M Lösung wird häufig in Zellen der Wurzelepidermis das Auftreten von Protuberanzen festgestellt, während die Kerne der Meristemzellen ausgeprägte Vakuolenbildung aufweisen. Gefärbtes Material liegt in Form von dünnen strukturlosen Bändern und Schollen an der Peripherie des Kernes (Abb. 6). Mit Hilfe der Phasenkontrast-Einrichtung sind innerhalb der Vakuolen die Nucleolen in ursprünglicher Grösse erkennbar. Es wurden auch Zellen mit Karyomeren beobachtet; hier dürfte es sich um rückgebildete C-Mitosen handeln, deren Chromosomen einzelne Karyomeren gebildet haben, die im Innern von zahlreichen Vakuolen erfüllt sind (Abb. 8).

Wirkt eine Lösung gleicher Konzentration 72 Stunden lang auf die Wurzeln ein, so kommt es in den meisten Zellen zu einem vollständigen oder fast vollständigen Verschwinden des feulgen-positiven Materials.

Die Beschreibung der mit Penicillin erzielten Effekte kann hier sehr kurz

gehalten werden, da bereits reichhaltiges Beobachtungsmaterial vorliegt (10), das mit unserem vollkommen übereinstimmt. Es soll nur über in der erwähnten Arbeit wenig beobachtete Ausstülpungen von ungefärbtem Material aus derart behandelten Kernen berichtet werden.

Innerhalb der Kerne sind grosse, z. T. irregulär geformte Vakuolen beobachtbar, welche sehr oft mit ungefärbten Vorwölbungen des Kernes in Zusammenhang stehen (Abb. 7). Selten wird der Kern von einer geschlossenen Schale des feulgen-negativen Materials umgeben. Gleichartige Erscheinungen können auch an Kernen ohne Protuberanzen erkannt werden.

Nach 24-stündiger Einwirkung von 2,4-D in $1 \cdot 10^{-2}$ M Konzentration werden zahlreiche Formen von Protuberanzen beobachtet. Man sieht Protuberanzen, die fast die Grösse des Kernes erreichen neben Zellen, deren Plasma mit unzähligen winzigen Körnchen und fadenförmigen Gebilden erfüllt ist. Andere Kerne wieder enthalten überhaupt keine Thymonucleinsäure (DNS) mehr und lassen nach Anfärbung mit Lichtgrün eine zarte Reststruktur erkennen. Grössere kugelige Protuberanzen enthalten im Innern selbst Vakuolen, was eine weitere Entmischung des beim Austritt vermutlich homogenen Materials wahrscheinlich macht (Abb. 9).

Die mit wässrigem Knoblauchextrakt erzielten Effekte haben wir bereits an anderer Stelle beschrieben (6). Im Gegensatz zu den anderen Testsubstanzen kann Knoblauchextrakt Protuberanzen bewirken, ohne dass dabei stärkere morphologische Veränderungen der Ruhekerne des Wurzelmeristems sichtbar werden. Die Protuberanzen in den Meristemzellen sind kleine faden- oder tröpfchenförmige Gebilde, während in den Zellen des Rindenparenchyms und der Epidermis vorwiegend grössere Kugeln sichtbar sind. Gelegentlich sind farblose, blasenförmige Ausstülpungen der Kerne zu sehen, die an ihrem der Kernoberfläche entgegengesetzten Ende von einer dünnen Lamelle feulgen-positiven Material umgeben sind (Abb. 10). Bei den Mitosen findet man eine starke Verklebung der Chromosomen, deren Inneres von zahlreichen kleinen Bläschen erfüllt ist.

Sakaguchi-Reaktion

Bei Präparaten unbehandelter Wurzeln findet man, dass die stärkste Reaktion auf arginin-reiche Eiweisskörper von den Nucleolen gegeben wird, während das Cytoplasma nur schwach gefärbt ist. Die Orangefärbung des Chromatins der Kerne liegt etwa in der Mitte; Chromosomen und Telophasekerne zeigen etwa die gleiche Farbtiefe wie die Nucleolen. Das Cytoplasma der Meristemzellen lässt — verglichen mit dem Plasma bereits differenzierter

Zellen — eine relativ hohe Argininkonzentration erkennen, die vermutlich durch den Gehalt an Ribonucleohiston, der für in Teilung befindliche Zellen charakteristisch ist (1), bedingt ist. Das Chromatin der Ruhekerne weist feinkörnige Struktur auf, die Nucleolen sind ohne den bekannten, durch schlechte Fixierung bedingten leeren Raum im Chromatin eingebettet (Abb. 1); an den Chromosomen der Mitosestadien ist eine leichte Quellung erkennbar (Abb. 16).

4-stündige Einwirkung von Knoblauchextrakt bewirkt keine sichtbare Veränderung der Ruhekerne der Meristemzellen. Kleine Protuberanzen, wie sie in analogen Feulgen-Färbungen beobachtet werden können, sind hier infolge der geringen Farbdifferenzierung nur in seltenen Fällen erkennbar. In Epidermiszellen, Zellen der Kalyptra und des Rindenparenchyms der Wurzel sind die optischen Verhältnisse günstiger; die Färbung des dünnen Plasmabelages ist gering, die Protuberanzen sind voluminöser und können innerhalb der Zelle als intensiv orange gefärbte Körper identifiziert werden. Mitosestadien bilden irreguläre Massen, an welchen einzelne Ausstülpungen noch die Chromosomen vermuten lassen, und sind sakaguchi-negativ. Aus den wechselnden Brechungsverhältnissen beim Höher- und Tieferdrehen der Mikrometerschraube des Mikroskops kann geschlossen werden, dass hier — verglichen mit dem umgebenden Plasma — ein optisch dünneres Material vorliegt. Beide Beobachtungen sind bemerkenswert, da in unbehandelten Kontrollpräparaten Meta- und Telophasechromosomen stark gefärbt sind und optisch dichter erscheinen. An der Grenze zwischen dem verklebten Chromosomenklumpen und dem Plasma kann ein stärker gefärbter Saum beobachtet werden (Abb. 5).

Im Gegensatz zu Knoblauchextrakt bewirken hohe Konzentrationen von Penicillin und LiNO_3 tiefgreifende Veränderungen der Ruhekerne. Es ist vor allem auffallend, dass die Grundmasse, welche den Hauptteil des ursprünglichen Kernraumes einnimmt, keine Sakaguchi-Reaktion gibt und gegenüber der Plasmafärbung als heller Körper hervortritt (Abb. 15). Sie ist entweder optisch leer oder weist seltener feine netzartige Strukturen auf, die jedoch nicht gefärbt sind, sondern sich nur durch unterschiedliche Brechungsverhältnisse hervorheben. In dieser Grundsubstanz sind stets wenige kleinere oder grössere stark gefärbte Körper eingebettet, die nicht in allen Fällen mit den Nucleolen identifiziert werden können. Gelegentlich führen feine Fäden von den grösseren Einschlüssen zur Peripherie des Kernraumes und stehen dort mit grösseren Schollen in Verbindung, die dem ungefärbten Körper aussen anliegen (Abb. 4) und ebenfalls stark gefärbt sind. Stets ist die ungefärbte Grundsubstanz des Kernes von einzelnen Schollen

oder auch einer lückenlosen Hülle von argininreichem Material umgeben, das teils als scharfbegrenzte Vorwölbung ins Plasma auftritt, teils auch ohne scharfe Abgrenzung ins Plasma übergeht; das Plasma solcher Meristemzellen zeigt dann eine stärkere Färbung als im Normalfalle. An Hand von Vergleichen mit entsprechenden feulgen-gefärbten Präparaten kann mit grosser Wahrscheinlichkeit angenommen werden, dass die sakaguchinegative Grundsubstanz DNS enthält, während die arginin-reichen Gebilde an der Kernperipherie und die eingeschlossenen Körper DNS-frei sind.

LiNO_3 in $3 \cdot 10^{-2}$ M Lösung und 2,4-D 10^{-2} M bewirken geringere Veränderungen in der Lokalisation der arginin-reichen Kernstrukturen (Abb. 2, 3, 17). Freie Protuberanzen in Epidermiszellen geben auch hier eine positive Sakaguchi-Reaktion.

Lebendbeobachtungen

Es wurden folgende Versuchsobjekte gewählt: Zellen der Staubfadenhaare von *Tradescantia virginica*, des Rindenparenchyms von *Allium cepa*-Wurzeln und der Innenepidermis von Zwiebschuppen von *Allium cepa*.

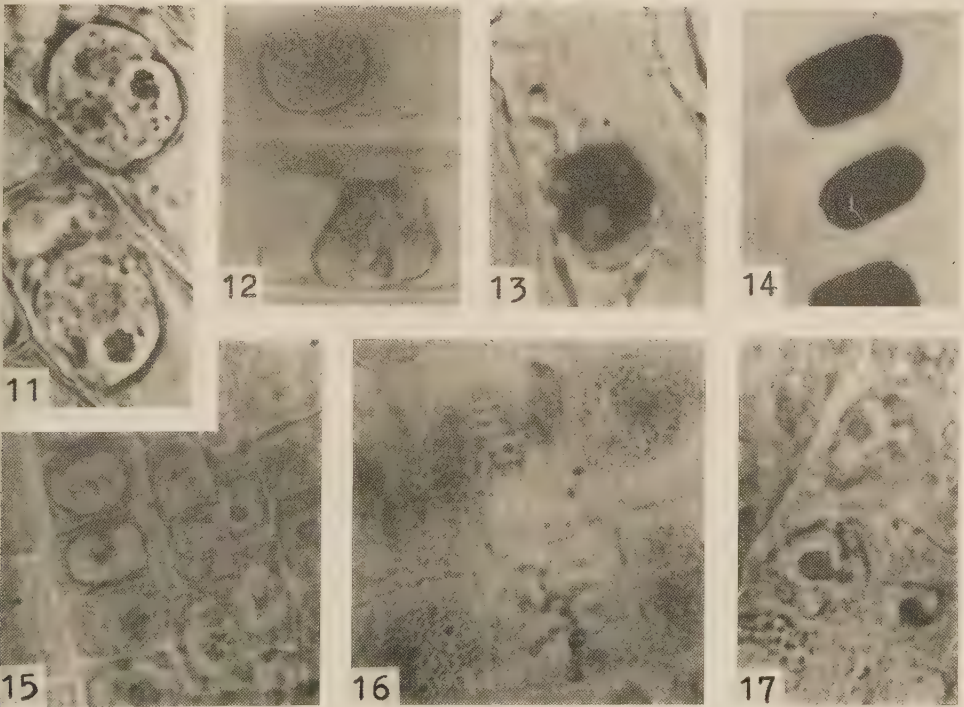
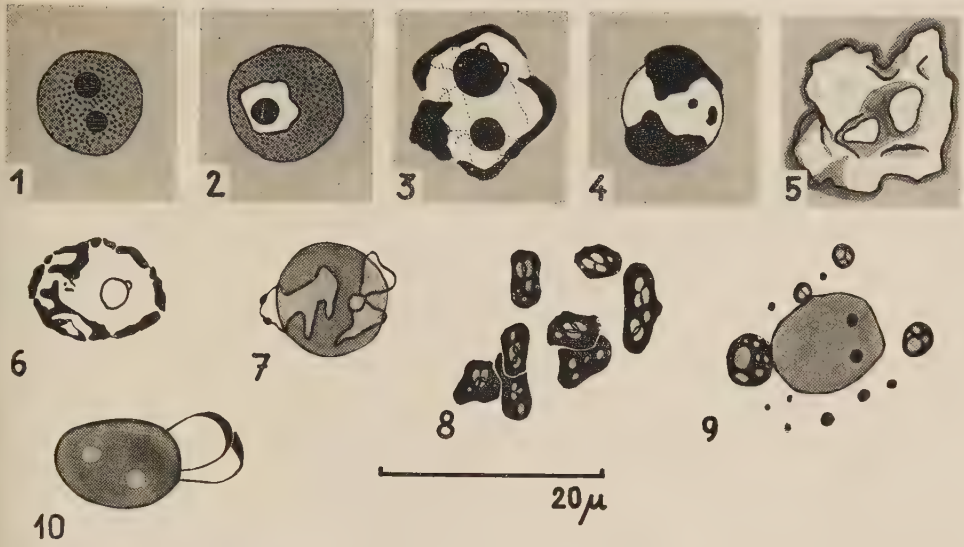
Die beschriebenen Objekte wurden möglichst schonend herauspräpariert, in einem Tropfen der zu untersuchenden Lösung auf einem Objektträger gebracht, unter Vermeidung von Druck ein Deckglas aufgelegt und zuletzt mit Paraffin umrandet. Auf die gleiche Weise stellten wir Kontrollpräparate in Leitungswasser her.

Die Staubfadenhaare von *Tradescantia* wurden jungen Blütenknospen entnommen und nur die plasmareichen Spitzenzellen untersucht. Unverletzte Zellen blieben in den Kontrollpräparaten mehrere Tage ohne sichtbare Kernveränderungen. Die relativ grossen Zellen des Rindenparenchyms der Wurzeln von *Allium cepa* ermöglichen es, durch Handschnitte genügend dünne Präparate herzustellen. Es wurden Längsschnitte knapp unter der Oberfläche der Wurzel, etwa 2 mm hinter der Wurzelspitze beginnend, gemacht. An anderen, auf die gleiche Weise hergestellten Präparaten gelingt es, durch leichtes Andrücken des Deckglases einige Kerne aus angeschnittenen Zellen herauszubefördern, wodurch unbeschädigte, frei in der Lösung

Sakaguchi-Reaktion: Die Farbintensität des Cytoplasmas wird bei den Abbildungen 1 bis 5 durch den grauen Untergrund angedeutet. Abb. 1. *Allium sativum*, unbehandelt. Abb. 2. *Allium cepa*, 2,4-D 10^{-2} M, 24 Std. Abb. 3. *Allium cepa*, LiNO_3 $3 \cdot 10^{-2}$ M, 24 Std. Abb. 4. *Allium sativum*, Penicillin 20 MIU, 4 Std. Abb. 5. *Allium sativum*, Knoblauchextrakt, 4 Std.

Feulgen-Reaktion: Abb. 6, 8. *Allium cepa*, LiNO_3 $3 \cdot 10^{-2}$ M, 24 Std. Abb. 7. *Allium sativum*, Penicillin 20 MIU, 4 Std. Abb. 9. *Allium cepa*, 2,4-D $1 \cdot 10^{-2}$ M, 24 Std. Abb. 10. *Allium sativum*, Knoblauchextrakt, 4 Std.

Abb. 11. *Allium cepa*, Zwiebelepidermis, Penicillin 20 MIU, 12 Min., Phasenkontrast $900 \times$. Abb. 12. *Allium cepa*, Zwiebelepidermis, Penicillin 20 MIU, Sakaguchi-Reaktion, $900 \times$. Abb. 13. *Allium cepa*, Zwiebelepidermis, Penicillin 20 MIU, Feulgen-Reaktion, Phasenkontrast, $900 \times$. Abb. 14. *Allium cepa*, LiNO_3 $3 \cdot 10^{-2}$ M, 24 Std. Feulgen-Reaktion, Phasenkontrast $950 \times$. Abb. 15. *Allium sativum*, Penicillin 20 MIU, 4 Std. Sakaguchi-Reaktion, $950 \times$. Abb. 16. *Allium sativum*, unbehandelt, Sakaguchi-Reaktion, $1000 \times$. Abb. 17. *Allium sativum*, LiNO_3 $3 \cdot 10^{-2}$ M, 24 Std. Sakaguchi-Reaktion $950 \times$.



schwimmende Kerne erhalten werden; diese eignen sich infolge günstiger optischer Verhältnisse sehr gut zum Studium der strukturellen Veränderungen. Die Innenepidermis der Zwiebschuppen wurde mit einer Pinzette vorsichtig abgezogen und ein 5×5 mm grosses Stück ausgeschnitten und untersucht.

Untersucht wurden folgende Lösungen: 2,4-D (0,025 M), Penicillin G (10 und 20 MIU) und Knoblauchextrakt. 1 M LiNO_3 -Lösung war ungeeignet, da die starke Plasmalyse der Zellen die Beobachtung stört.

Bei allen Versuchen waren bestimmte charakteristische Veränderungen der Zellkerne zu beobachten. Die einzelnen Testsubstanzen unterschieden sich dabei im Wesentlichen durch die für die Effekte benötigte Einwirkungs-dauer. Die Entstehung von Protuberanzen konnte nur in den seltensten Fällen beobachtet werden; es kann aber indirekt geschlossen werden, dass nach dem öfters beobachteten Aufplatzen von Kernen Protuberanzen gebildet wurden. Das weitere Schicksal der dabei freiwerdenden Substanz entzog sich infolge ungenügender Differenzierung im Phasenkontrastmikroskop meist der Beobachtung.

Es folgt die Beschreibung einzelnen besonders charakteristischer Effekte:

Nach etwa 20 Min. langer Einwirkung einer Penicillinlösung (20 MIU/100 ccm) auf einen isolierten Kern des Rindenparenchyms der Wurzel von *Allium cepa* bildet sich um jeden der Nucleolen eine helle Vakuole, die sich im weiteren Verlaufe der Einwirkung vergrössert. Innerhalb der Vakuolen stehen die Nucleolen unter dem Einfluss der Brown'schen Molekularbewegung; dies deutet ohne Zweifel auf eine dünnflüssige Konsistenz des Vakuoleninhaltes. Etwa 60 Min. nach Herstellung des Präparats verblassen die inzwischen gröber gewordenen Chromatinstrukturen des Kernes zu verwaschenen, kaum erkennbaren Flecken; die Vakuolen werden undeutlich und verschwinden zuletzt. Die Nucleolen liegen nunmehr als dunkle Körper unbeweglich in der hellen, homogen erscheinenden Kernsubstanz eingebettet. Der Kern lässt eine Volumsvermehrung erkennen, die durch Eindringen von Flüssigkeit erklärbar ist. Bei genauer Betrachtung können im Innern des Kernes feine, an der Grenze der optischen Auflösung liegende perlschnurartig aneinandergereihte Körnchen erkannt werden, die an die Chromomeren der Leptotäanchrosomen erinnern.

Eine eigenartige Entmischungserscheinung wurde bei den Zellen des Rindenparenchyms von *Allium cepa*-Wurzeln nach Behandlung mit der gleichen Lösung an Ausstülpungen der Kerne beobachtet. Ähnlich einer grossen Blase dringt eine Kernausstülpung in die Zellsaftvakuole ein und nimmt — vermutlich durch die Spannung der Kernmembran — die Form einer Halbkugel an. An ihrer Oberfläche hebt sich eine mit der Zeit dicker

werdende Lamelle ab, die als optisch dichter erscheint. Bei weiteren Vordringen in das Plasma wird die Lamelle undeutlich; ein Platzen wurde nicht beobachtet.

Nach 40 Min. Einwirkung einer Knoblauchextraktlösung auf Staubfadenhaare von *Tradescantia* entstehen Nucleolarvakuolen. Innerhalb des Kernes bilden sich helle Gänge, die mit den Vakuolen um die Nucleolen in Verbindung stehen und sich auch als Vorwölbungen der Kerne ins Plasma erstrecken. Befindet sich in der Nähe des Kernes eine Zellsaftvakuole, so kann die Kernausstülpung diese zur Seite drängen oder in sie eindringen. Nach 2-stündiger Versuchsdauer liegen die Kerne als glasige, stark lichtbrechende Kugeln in den Zellen; in ihrem Innern lassen sich keine Strukturen beobachten.

Diese Effekte sind weitgehend denjenigen analog, welche an dem gleichen Objekt durch 10 MIU Penicillin hervorgerufen werden.

Auch an Chromosomen der Mitosen von *Tradescantia* wird nach Behandlung mit 10 MIU Penicillin bei Phasenkontrastbeobachtung eine Aufhellung festgestellt. Die vorerst dunklen Chromosomen verkleben unter gleichzeitiger Aufhellung; für einige Sekunden werden sie infolge gleicher Helligkeit im Plasma unsichtbar, nach 70 Min. Versuchsdauer liegen sie dann als helle Körper im nunmehr dunkler erscheinendem Plasma vor.

Nach 2-stündiger Einwirkung von 2,4-D auf Kerne der Zwiebelepidermis beobachtet man ein Hin- und Hertanzen der Nucleolen infolge Brown'scher Molekularbewegung, was ebenfalls durch Verflüssigung der Kernbezirke um die Nucleolen bedingt sein dürfte. In weiterer Folge ist gleichzeitige Aufhellung des übrigen Chromatins und Verblasen der Strukturen erkennbar.

Nach Einwirken einer Lösung von 20 MIU/100 ccm Penicillin G auf Präparate der Zwiebelepidermis konnten wenige Min. nach der Herstellung nur mehr stark beschädigte Kerne beobachtet werden. Die Veränderungen mussten unmittelbar nach dem Einbringen des Objekts in die Lösung erfolgt sein. Unter Verzicht auf die Paraffinumrandung wurde deshalb ein Präparat in wenigen Sekunden fertiggestellt und sofort beobachtet. Nach 2–3 Min. wurde die Körnchenstruktur der Kerne deutlicher, und die Nucleolen dunkler. Etwa 4 Min. nach Beginn der Präparation bildeten sich Vakuolen um die Nucleolen, Verzweigungen drangen in den übrigen Kernraum ein und füllten diesen bald darauf unter gegenseitiger Verschmelzung aus. Inzwischen verblasen sämtliche Kernstrukturen, auch die Nucleolen wurden für kurze Zeit unsichtbar; der Kern lag dann als kaum vom Plasma unterscheidbares Gebilde in der Zelle. Die beschriebenen Veränderungen im Kern gingen innerhalb 1 Min. vor sich; man kann den Vorgang am ehesten als

ein Einschmelzen der Kernstrukturen bezeichnen. Nach weiteren 3 Min. wurden im Kern dunkle Schollen sichtbar, der restliche Kerninhalt hellt inzwischen stark auf, während das den Kern umgebende Plasma etw dunkler wird (Abb. 11).

Werden die Zellen in diesem Augenblick fixiert und der Sakaguchi-Reaktion unterworfen, erfolgt starke Anfärbung der an der Kernoberfläche liegenden Schollen sowie der Nucleolen im Innern; es färben also gerade jene Elemente an, die bei der Lebendbeobachtung im Phasenkontrast-Verfahren dunkel abgebildet wurden. Auch das den Kern umgebende Plasma reagiert stärker als das übrige Plasma der Zelle (Abb. 12).

Werden die gleichen Präparate der Feulgen'schen Nuclealreaktion unterworfen, lässt sich nur in der bei der Lebendbeobachtung hell erscheinenden Grundsubstanz des Kernes die Anwesenheit von DNS erkennen. Wie auf Abb. 13 ersichtlich ist, befindet sich an der Aussenseite des Kernes feulgen-negatives Material, dass sich infolge der Phasenkontrastwirkung als dunklere Partie abhebt.

Ein Aufplatzen von Kernen des Rindenparenchyms von *Allium cepa*-Wurzeln wurde nach Einwirkung von Knoblauchextrakt öfters beobachtet. Vorher findet starke Volumsvermehrung der Kerne unter gleichzeitiger Aufhellung statt.

Färbung mit Methylgrün

Wir färbten mit Methylgrün-Pyronin nach einer von Chayen (2) entwickelten Methode, weiters nach Kurnick (7) mit Methylgrün allein. Als Objekte wurden Schnitte von *Allium cepa*-Wurzelspitzen, welche mit den in Frage kommenden Substanzen behandelt waren, und entsprechend unbehandelte Kontrollen verwendet. Die erhaltenen Ergebnisse sind in der Tabelle I zusammengefasst.

TABELLE I

Behandlung	Methylgrün-Pyronin		Methylgrün	
	Chromatin	Plasma	Chromatin	Plasma
Unbehandelt	blau	hellrot	blaugrün	0
4 ^h Knoblauchextrakt	violett	hellrot	blaugrün	0
24 ^h 2,4-D $1 \cdot 10^{-2}$	karmin	hellrot	0	0
4 ^h LiNO ₃ 1 M	karmin	hellrot	0	0
24 ^h LiNO ₃ $1 \cdot 10^{-2}$ M	violett	hellrot	schwach blaugrün	0
4 ^h Penicillin 20 MIU.	karmin	hellrot	0	0

Beide Versuchsreihen zeigen übereinstimmende Resultate, wenn man annimmt, dass bei fehlender oder schwacher Reaktion des Chromatins mit Methylgrün eine entsprechend starke Adsorption von Pyronin erfolgt und dabei Mischfarben entstehen.

Alle Lösungen, die in den Wurzelzellen starke Veränderungen der Ruhekernstrukturen bewirken und Chromatinaustritt verursachen, führen gleichfalls zu einem negativen Ausfall der Methylgrünfärbung, d.h. sie verursachen vermutlich eine Depolymerisation von DNS (7, 14).

DISKUSSION

Die Vielfalt der Versuchsergebnisse mag auf den ersten Blick verwirrend erscheinen; bei genauerer Betrachtung finden sich jedoch bei allen Versuchen charakteristische gemeinsame Effekte, die eine weitgehende Einheitlichkeit der erzielten Veränderungen wahrscheinlich machen.

Die Vorgänge im Zellkern nach Behandlung mit den genannten Substanzen lassen sich in zwei Phasen unterteilen. Für die erste Phase ist die Bildung von Vakuolen in den Zellkernen charakteristisch. Sie entstehen vorwiegend um die Nucleolen herum und vergrößern sich im weiteren Verlaufe der Einwirkung. Der Vakuoleninhalt ist dünnflüssig, enthält keine DNS und wahrscheinlich auch keine arginin-reichen Eiweisskörper. Es kommt zur Bildung von optisch leeren Gängen, welche den Kernraum durchziehen, mit den Nucleolarvakuolen in deutlich erkennbarem Zusammenhang stehen und von der gleichen Flüssigkeit erfüllt sein dürften. DNS-haltige Kernstrukturen geben in der ersten Phase stets eine stark positive Sakaguchi Reaktion.

Im weiteren Verlauf der Einwirkung der höchsten untersuchten Konzentrationen von Penicillin und LiNO_3 wird ein vollständiges Einschmelzen der Chromatinstrukturen beobachtet; die Lebendbeobachtung lässt erkennen dass dabei gleichzeitig die Flüssigkeit der Nucleolarvakuolen vom übrigen Kerninhalt aufgesaugt wird. Die Grundsubstanz des Kernes wird dabei gel-artig und glasig. Im Phasenkontrastmikroskop hellen die vorher dunklen Kerne stark auf, erscheinen homogen und lassen erst einige Zeit darauf die Nucleolen als nunmehr dunklere Körper erkennen. Den gesamten beschriebenen Erscheinungskomplex, beginnend mit der Rückbildung der Kernvakuolen, möchten wir als die zweite Phase bezeichnen. Aus den cytochemischen Reaktionen ergibt sich die interessante Tatsache, dass in der zweiten Phase eine Trennung von DNS und der arginin-reichen Eiweisskomponente (Histon) stattfinden muss (Abb. 11, 12, 13). Die im Phasen-

kontrastmikroskop hell erscheinende Hauptmasse der Kerne enthält DNS, während die Schollen an der Kernperipherie keine DNS, dafür aber argininreiche Substanzen enthalten. Es ist wahrscheinlich, dass frei gesetzte DNS von hochviskoser Konsistenz das Aufsaugen der Vakuolenflüssigkeit bewirkt. Während nun die DNS-haltige Substanz den ursprünglichen Kernraum nahezu zur Gänze einnimmt, wird neben den argininreichen Schollen an der Kernperipherie noch eine erhöhte Arginkonzentration in dem den Kern umgebenden Plasma beobachtet. Es scheint eine Diffusion argininreicher Kernproteine in das Plasma stattgefunden zu haben.

Bei den Mitosestadien beobachtet man Veränderungen, die der zweiten Phase analog sind; im Phasenkontrastmikroskop kann an *Tradescantia* Aufhellung und Quellung der Chromosomen erkannt werden; diese Erscheinungen sind mit Austreten von argininhaltiger Substanz verbunden. Diese tiefgreifenden chemischen Veränderungen der Mitosestadien finden sich schon bei Konzentrationen bei welchen die Ruhekerne des gleichen Präparates noch kaum eine leichte Schädigung erkennen lassen. Dies lässt sich entweder durch eine gewisse Schutzwirkung der Kernmembran oder durch verschiedene Angreifbarkeit des Chromatins in den Chromosomen und dem Ruhekern deuten.

Da die Protuberanzen in allen untersuchten Fällen stets DNS und argininreiches Protein enthalten, kann angenommen werden, dass ihre Entstehung vor der chemischen Entmischung des Chromatins — also in der ersten Phase — erfolgt ist.

Levan und Tjio (10) vermuteten bereits, dass unter diesen Bedingungen infolge des erhöhten osmotischen Druckes innerhalb der Kerne ein Auspressen des Chromatins erfolgt. Unsere Ergebnisse unterstützen diese Theorie. Wir konnten oftmals bei Lebendbeobachtungen eine Vergrößerung der Kerne erkennen; die entstandenen flüssigkeitsgefüllten Nucleolarvakuolen scheinen einen Druck auf die äusseren Kernpartien auszuüben. An der Kernperipherie liegende Vakuolen traten als pralle Ausstülpungen des Kerns hervor und können kleine Zellsaftvakuolen zur Seite drängen und in grössere eindringen. In ihrem Inneren müsste also ein stärkerer osmotischer Druck herrschen als in den Zellsaftvakuolen. Es wäre ohne weiteres denkbar, dass zentral im Kern liegende Vakuolen ein Auspressen des peripheren Chromatins zur Folge haben. Vakuolenausstülpungen können selbst feulgenpositives Material ins Plasma bringen, wie aus Abb. 10 klar ersichtlich ist.

Als Ursache für die Entstehung hoher osmotischer Werte innerhalb der Kerne muss wohl eine Depolymerisation der DNS angenommen werden, was auch durch den negativen Ausfall der Methylgrünfärbung bestätigt

wird. Neben osmotischen Kräften möchten wir jedoch auch Quellungskräfte in Betracht ziehen, die durch Hydratation des freigesetzten DNS-Gels entstehen könnten.

Es ist aber unwahrscheinlich, dass die verschiedenen Substanzen allein eine Depolymerisation der DNS bewirken; es ist eher zu erwarten, dass hier spezifische Kern- bzw. Zellenzyme beteiligt sind.

ZUSAMMENFASSUNG

Substanzen, welche den Austritt von Chromatin aus Zellkernen verursachen, und zwar Penicillin G, LiNO_3 , 2,4-Dichlorphenoxyessigsäure und ein wässriger Extrakt aus Zwiebeln von *Allium sativum*, wurden auf Wurzeln von *Allium cepa* und *Allium sativum* einwirken gelassen. Schnitte derartig vorbehandelter Wurzeln wurden der Feulgen-Reaktion und der Sakaguchi-Reaktion unterzogen sowie nach Kurnick mit Methylgrün gefärbt. Es wurden auch Lebendbeobachtungen während der Einwirkung der Lösungen auf Staubfadenhaarzellen von *Tradescantia virginica*, Epidermiszellen der Zwiebelschuppen von *Allium cepa* und Zellen des Rindenparenchyms von *Allium cepa* Wurzeln vorgenommen.

In allen untersuchten Fällen konnte in den freien Protuberanzen Thymonucleinsäure (DNS) und eine arginin-reiche Substanz, vermutlich Histon, nachgewiesen werden. Die Ruhekerne zeigen Strukturveränderungen, Vakuolenbildung, gelegentlich auch Ausstülpungen eines DNS-freien Materials. Bei stärkerer Schädigung der Kerne wurde eine chemische Entmischung des Chromatins festgestellt, es handelt sich hierbei vermutlich um eine Spaltung des Thymonucleohistons in seine beiden Komponenten. Ausser Knoblauchextrakt bewirken alle untersuchten Substanzen in höheren Konzentrationen eine Depolymerisation von DNS, die durch das Ausbleiben der Methylgrünfärbung angezeigt wird.

Zeitlich können zwei Phasen unterschieden werden. In der ersten Phase bilden sich Flüssigkeitsvakuolen in den Zellkernen, in der zweiten Phase findet Einschmelzung der Kernstrukturen und wahrscheinlich auch Entmischung des Chromatins statt. Die Protuberanzen dürften in der ersten Phase entstehen, als Ursache wird ein Auspressen des peripheren Chromatins infolge osmotischer oder Quellungskräfte angenommen.

Die Tatsache, dass chemisch so verschiedene Substanzen weitgehend übereinstimmende Wirkungen hervorrufen, lässt auf eine Unspezifität der Effekte schliessen; es kann angenommen werden, dass die von diesen Substanzen verursachte Abtötung der Zellen, bei der keine Fixierung erfolgt,

zelleigene Mechanismen auslöst, die in allen beschriebenen Fällen gleichartig funktionieren.

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THE RELEASE OF MINUTE GRANULE-CONTAINING ELEMENTS FROM THE EOSINOPHIL CELLS OF THE BLOOD

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THE different cellular elements of the blood have been the object of detailed studies ever since they were first recognised. Few fields of investigation have been so thoroughly studied as blood and its formed elements, and numerous articles are still appearing. Although the histogenesis and the morphology of the leucocytes have thus been studied intensively, little is known of their fundamental, physiologic functions.

The eosinophil leucocytes in normal blood vary in number from 2-5 per cent of the total leucocyte count. The nucleus generally has two oval lobes connected by a thin chromatin thread. The cytoplasm contains a number of granules of fairly uniform size, and they stain brilliantly with acid dyes. For this reason the cells are also called oxyphils or acidophils.

The eosinophils are less motile and less phagocytic than the neutrophils. They are greatly increased in number in allergic conditions and in many parasitic infections. The staining qualities of the granules have led to a theory that they were ingested particles of red blood corpuscles or particles containing haemoglobin derived from degenerating muscle cells (14, 12). If the granules are haemoglobin derivatives their presence in the cells is not yet fully explained.

Advances in electron microscopy have widened the field of morphology. With the aid of this new technique the fine structure of the elements of the blood has been studied by a number of workers (2, 3, 4, 5, 13, 1, 8, 10).

Electronmicroscopical studies have been carried out on, among other things, human blood platelets (6, 7). In a great number of preparations eosinophil leucocytes were found and a closer study of these cells was considered important.

MATERIALS AND METHODS

A modification of a method reported by Bessis and co-workers (2, 4), Rebuck (13), Bernhard, Braunsteiner *et al.* (1) was used. A glass slide is dipped in a formvar solution to get a thin film on the glass. The formvar film can easily be loosened and transferred first to a water surface and then from there to another narrow slide, on which is placed a specimen screen for electron microscopy. After drying the screen lies between the glass slide and the formvar film.

Human blood was obtained by venous puncture and caught in a glass tube containing a small quantity of heparin. The special slide for electron microscopy was then immersed in the blood and placed vertically to avoid the cells settling upon the formvar film just by sedimentation. The tubes with the blood were then put into an incubator with a temperature of 37°C. The period of incubation varied from a few minutes to two hours, whereafter the slides were taken out, rinsed in a physiological saline solution or Tyrode's solution in order to remove the erythrocytes and fixed in a buffered 1 per cent osmiumtetroxide solution according to Palade (11). The slides were then rinsed in distilled water and put to dry in the air. After drying it was easy to loosen the specimen screen with the formvar film. The specimens were examined in a Siegbahn-Schönander electron microscope.

RESULTS

Human leucocytes are far from ideal electronmicroscopical objects. Due to the density of the central part containing the nucleus, the electron beam is transmitted only with difficulty, and a close study of the fine structure is here impossible.

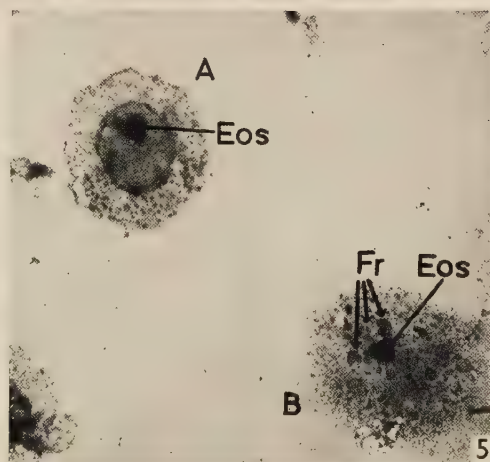
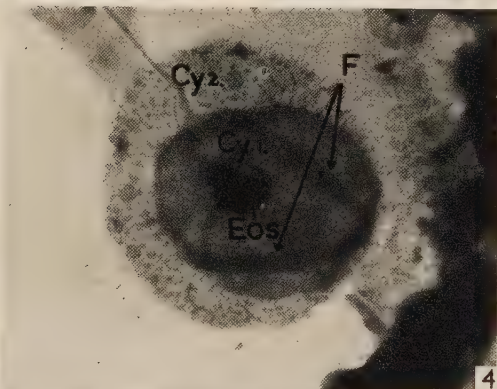
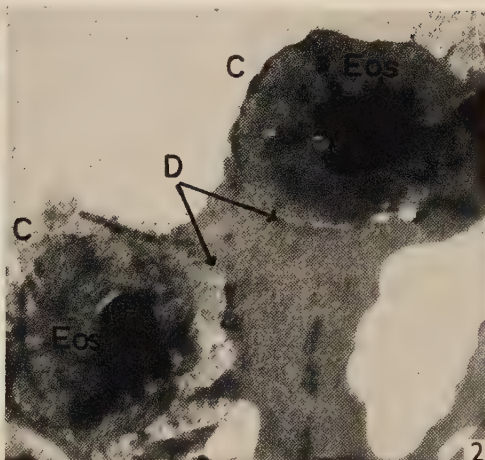
Fig. 1. Eosinophil leucocyte from homo blood. The central part is too dense to be penetrated by the electron beam. A number of eosinophil granules are seen. In the upper right hand corner (arrow) a small, circular corpuscle containing a single granule is seen budding off. Electronmicrograph. Fix: Buffered osmic acid. Magnification about 10,000 ×.

Fig. 2. From the periphery of an eosinophil leucocyte a cytoplasmic protrusion is seen. D shows a line of demarcation between the cytoplasm of the cell and the small corpuscles (C) which are just being isolated. The dense eosinophil granules (Eos) are distinctive. Electronmicrograph. Fix: Buffered osmic acid. Pt/Pd shadowed preparation. Magnification about 20,000 ×.

Fig. 3. An isolated corpuscle from an eosinophil leucocyte. In the center one or more granules densely packed together (Eos). Surrounding the granules is the cytoplasm (Cy). The remains of the connection between the cell and the corpuscle is seen (arrow). Electronmicrograph. Fix: Buffered osmic acid. Pt/Pd shadowed. Magnification about 20,000 ×.

Fig. 4. An isolated eosinophil corpuscle undergoing lysis. The central granular mass is intact (Eos). The cytoplasm (Cy 1) is enveloped by a membranous structure of which we here see marked folds (F) as the membrane falls together. Outside the membrane droplets or granules of cytoplasmic matter (Cy 2) are clearly seen emerging from the corpuscle. Electronmicrograph. Fix: Buffered osmic acid. Pt/Pd shadowed. Magnification about 20,000 ×.

Fig. 5. Two disintegrating eosinophil corpuscles. The granules (Eos) seem to be unaffected. A shows an early stage with a dense central cytoplasmic part still intact, while in B an explosive disintegration has occurred. Notice the larger fragments in B (Fr) which seem to be the remains of the central cytoplasm. Electronmicrograph. Fix: Buffered osmic acid. Magnification about 12,000 ×.



Surrounding the central part is the cytoplasm of the cell spreading out in a thin layer. Here a great number of round granules are seen. They are much larger than those of the neutrophils but exceed these in number. The granules are as a rule completely electron impermeable, their size varies from 0.3–0.6 microns.

From the border of many eosinophil cells small, granule-containing elements are seen budding off (Fig. 1). These small bodies have a circular form and contain one or several granules, densely packed together in their center, surrounded by cytoplasm. The diameter of these small bodies varies but little around 2.5–3 microns. Different stages in their development are seen. At the border of a cell a little circular mass of cytoplasm with granules starts to withdraw from the cell. In the beginning a thick strand of cytoplasm connects it to the cell. This strand becomes thinner as the body withdraws. Suddenly a line of demarcation appears between the cytoplasm of the cell and the cytoplasm around the granules (Fig. 2) and finally the corpuscle is isolated. Of great interest is the fact that there seems to be a thin membrane surrounding the corpuscles (Fig. 4).

These small elements are intact only for a short time. After being isolated they readily disintegrate (Figs. 4 and 5). The disintegration seems to occur by way of some sort of lysis. It is extremely difficult to determine whether this is an artefact due to preparation or a normal function. In pictures obtained (Fig. 4) a folded membranous structure is seen enclosing a dense central core of cytoplasm in the center of which the eosinophil granules appear. The periphery shows a great number of small droplets or granules of cytoplasmic matter which seem to have emerged from the corpuscle. These small granules have a diameter of about 500–1000 Å. In other pictures the whole corpuscle has disintegrated explosively, the dense central part breaking up into larger fragments measuring about 1700–3000 Å across. The eosinophil granules in the center are mostly unchanged but have sometimes split into smaller parts. Rarely is the granule dissolved before the corpuscle disintegrates. After disintegration the granules are probably dissolved, as hardly any of them are found lying isolated on the formvar film.

A paper in press (6) deals with the disintegration of human blood platelets after taking up fine quartz particles. The disintegration of the thrombocytes was increased after taking up these fine particles. In a great many of the preparations eosinophil cells were found. After adding suspensions of fine particles of quartz, titaniumdioxide a.o. the tendency of the eosinophils to yield these small corpuscles as well as the disintegration of the latter was greatly increased.

DISCUSSION

Practically nothing is known of the function of the eosinophil cells of the blood. Their capacity for phagocytosis is small. They are said to participate in the detoxification of substances arising at the breakdown of albuminous products as well as in the direct defensive mechanism against foreign proteins (9). Their eventual connection with haemoglobin derivatives has been mentioned as well as their appearance in great numbers in allergic conditions and parasitic infections.

Morphological evidence for functional conditions of the cells of the blood has been scarce. Although the experiments here accounted for have been made *in vitro*, it seems probable that the elements described above are closely connected with the metabolism or function of the eosinophil cells. The nature of these small elements is unknown. The investigations are continued and further reports will be given.

SUMMARY

A brief description of the morphology of the eosinophil leucocytes is given. An electronmicroscopical study of these cells has shown them to release minute granule-containing corpuscles, surrounded by a membranous structure. The corpuscles disintegrate by some form of lysis. They may be important for the function of the cells.

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AN ELECTRON MICROSCOPE STUDY OF CARDIAC MUSCLE¹

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THIS paper reports an exploratory study with the electron microscope of cardiac muscle from a number of vertebrate species. Some of the recent improvements in fixation and sectioning techniques for electron microscopy have been applied (18, 20, 21, 24). Efforts have been made in this study to prepare tissue so as to preserve as nearly as possible the natural relationships of the constituent elements of cardiac tissue.

A considerable literature has appeared in reference to the electron microscopy of skeletal muscle, with less relating to cardiac muscle (3, 14, 15, 16).

The picture of heart muscle that has resulted from this study reiterates the close structural relationship long known to exist between cardiac and striated skeletal muscles. Such a similarity has been demonstrated in electron micrographs of fragmented muscle as well as in sectioned tissue. The observations reported here confirm the general concept of cardiac tissue given to us by the early histologists and presents some new data regarding the sarcosomes, the Z band, and the myofilaments of cardiac muscle.

MATERIALS AND METHODS

In preparing the tissue two techniques have been used: those of fragmentation and of sectioning. Samples from more than 200 hearts have been examined, from cat, dog, guinea pig, human, frog, snake, turtle, and chicken. The last (*Gallus*) was found to be most useful and served as the principal source of the material in this study.

Material for sectioning was obtained from sodium pentobarbital anaesthetized animals. The living heart was fixed while still beating by *in situ* interstitial injection of 2 per cent osmium tetroxide in Michaelis' veronal buffer adjusted to pH 7.2 to 7.4 (21). Small pieces of fixed tissue were then carefully dissected out and immersed in the fixative solution for four hours. Following this, the samples were washed in distilled water, dehydrated rapidly through a series of graded alcohols,

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transferred to monomeric n-butyl methacrylate, and embedded by polymerization of the methacrylate, either with a catalyst method of Newman, Borysko, and Swerdlow (20) or by exposure to ultra-violet light.¹ Some of the blocks were immersed during the dehydration process in 0.001 M phosphotungstic or phosphomolybdic acid in 70 or 95 per cent ethyl alcohol in order to increase the contrast of the electron micrographs.

Sectioning was performed on a Spencer No. 820 rotary microtome, using glass knives similar to those described by Latta and Hartman (18). The sections were advanced by the thermal expansion method (20). Sections were picked up from a 35 per cent alcohol surface onto stainless steel or copper grids previously coated with a thin film of Formvar.

Fragmented material was prepared from formalin-fixed or lyophilized pieces of heart muscle. After fixation, the tissue was fragmented either in a glass homogenizer or in a micro-blender, washed by centrifugation, and a drop of the resulting suspension was placed on the coated grids and allowed to dry.

Shadowing with heavy metals, usually nickel or uranium, was carried out occasionally, especially with the fragmented material, using the techniques described by Williams and Wyckoff (36). The sectioned material was prepared for shadowing by first removing the embedding plastic by immersion in toluene or chloroform.

The electron microscope used was an RCA-EMU with a 0.001 inch objective aperture. Its magnification was calibrated with polystyrene latex (1,8) for every important series of micrographs. The original magnification of the micrographs ranged from 3,000 to 10,000. Further enlargement was obtained by photographic means.

RESULTS

Structure of the myofibrils. Cardiac fibers are too gross in structure to be studied conveniently in the electron microscope, although their borders and components can readily be recognized in sectioned material. The most conspicuous feature in electron micrographs of sectioned heart muscle is the myofibril. These are definite units of structure, but in contrast to those of skeletal muscle, they display abundant anastomoses and branchings which are readily visualized in Figs. 1 and 3. The branchings occur in all planes, but a general parallel orientation is maintained. A myofibril may divide into two, each branch becoming separated from the other by sarcoplasmic constituents, only to recombine again within a few micra into a single fibril. This braided and interlacing myofibrillar pattern is most frequently seen. Occasionally, a more uniform configuration is observed, which resembles more nearly that of skeletal muscle, but which still presents a greater abundance of branching than does skeletal muscle (5).

¹ Polymerization of the methacrylate with ultra-violet light was accomplished conveniently by placing blocks of tissue into gelatine capsules containing uncatalyzed monomer plastic and supporting the capsules about 6 inches in front of a commercial 275 watt sun lamp (General Electric) in a current of cooling air. Polymerization usually occurred in 8-12 hours.

Figs. 1 and 2 demonstrate the appearance of typical myofibrils with their characteristic bands.

The myofibrils vary considerably in width, ranging from 0.2 micra to 10 micra, and all present evidence of cross-stripping. They are divided by the Z bands into structural units, the sarcomeres, which show considerable inconstancy in length. This can be attributed to the state of contraction, to differential stretching during preparation, and to species differences. In the present material, the sarcomere length of many species varies from 1.1 to 2.4 micra.

Myofilaments. The myofibrils are closely packed with much finer thread-like structures, the myofilaments, aligned longitudinally in parallel array, and passing occasionally in bundles from one myofibril to another in the branchings of the myofibrils mentioned above (Fig. 3). Fusion or branching of the myofilaments themselves has not been observed. No central core free of filaments has been seen in myofibrils (19, 25).

The myofilaments present periodically recurring structural features which are apparent in both the fragmented and sectioned material. In fragmented material the diameter of the filaments measured between 150 and 250 Å. Longitudinally along the filaments there seems to be a periodic accumulation of material, each unit measuring 320 to 480 Å in length, with a mean length of approximately 400 Å. In sectioned heart muscle, the myofilaments appear finer and considerably more uniform in regard to their diameters, which persistently approximate 100 Å. A longitudinal periodicity of 220 Å has been observed. At approximately the same intervals, minute interfilamentary bridges join neighboring filaments (D, Fig. 5). Similar fine periodic cross-bridging has been described in sectioned and fragmented skeletal muscle (13, 5). A 400 Å periodicity has been described previously in fragmented skeletal (10), cardiac (4), and smooth muscle (35). The difference in the appearance of myofilaments prepared by fragmentation and sectioning may be related to the presence or absence of interfilamentary material, which possibly is removed in most fragmented muscle preparations.

The relationship between the myofibril and connective tissue elements at the myo-tendonal junction, has not been elucidated in this study. Figure 7 is a section of chicken papillary muscle in which a projecting portion of the muscle is in very close contact with a large bundle of fibrils which are identified as collagen on the basis of their characteristic periodicity (29). A sudden transition can be seen between the myofilaments and the collagenous fibrils, but direct continuity cannot be seen in this micrograph. There is no distinguishable membrane separating the two types of fibers. The orientation of

the myofilaments at the tip of the projecting myofibrils is directed towards the collagen bundles.

Cross bands. In comparison with skeletal muscle the cross banding is less prominent. However all the bands described in skeletal muscle have been identified in the present cardiac material, though usually one or more of the characteristic bands is lacking. For example, frequently the Q and J bands cannot be distinguished, the myofibrils having a uniform appearance along their entire length, except for their course through the Z bands (Fig. 3). At other times, the area of the J band appears to show little evidence of the presence of the myofilaments, with only a few strands of myofilaments connecting one Q band to the Q band of the next sarcomere (Fig. 6). Under these circumstances the Z band may be absent also, or only traces of it may be seen. The longitudinal alignment of the individual sarcomeres, however, is maintained, even in the absence of visible continuity of the myofilaments. Neither the nature nor the exact disposition is known of the material which accounts for the increased density and the prominence of the myofilaments in the region of the Q band in cardiac muscle. However, Hanson and Huxley (11) have brought forth evidence that in striated skeletal muscle myosin may be preferentially localized in the Q.

The H, or clear band, which bisects the Q, is very faint or absent in strongly contracted muscle. However, in relaxed or partially contracted muscle it is often observed in the center of the Q band as a clearing in which myofilaments are more sharply outlined (Figs. 1 and 4). Its length varies from 0.1 to 0.3 micron. The H band may in turn be bisected by the M band, which is a delicate fine line crossing the myofibril (M, Fig. 1). It was seen infrequently in this material.

The N band can be seen in some instances as a fine dark line crossing the J band close to the Z. The portion of the J band lying between the N and Z bands is the E band. In the present study, the N and E bands were infrequently observed.

Z band. The Z band or telophragma divides the myofibrils into structural units, the sarcomeres. The Z bands are composed of granular material in which none of the characteristic periodicity of collagen is observed. The width of this band in electron micrographs varies between 0.05 and 0.30 micron, most of them being between 0.10 to 0.15 micron. Occasionally distinctly double Z bands are seen (Fig. 8). The lateral alignment of the sarcomeres from myofibril to myofibril is not dependent upon the presence of an intact telophragma, nor upon the continuity of this band between the myofibrils. Fig. 1 shows that even in the areas where the striations of the

myofibril are in good alignment, the Z bands do not cross the sarcoplasmic interval between them. Instead they appear to become dissipated in the sarcoplasmic reticulum, or to come into close contact with the sarcosomes. When these bands are continuous between adjacent myofibrils the portion crossing the sarcoplasm is usually very tenuous. The close association of the Z bands with sarcosomes is a very striking feature in many of the micrographs (Fig. 1). Often there appears to be direct continuity of material between the two structures. In other areas a more tenuous connection is seen, resembling fine strands of sarcoplasmic reticulum.

The Z band varies considerably in appearance in sectioned material. It may be seen as a prominent dense line (Fig. 1), as a thin tenuous strand (Fig. 6), or may be almost completely absent (Fig. 6). In the latter situation, small irregular granules can be seen occupying the site. Occasionally they are greatly widened and dense (Fig. 8). Such an appearance may be the result of an intense contraction (30). The shortening of the sarcomeres in this micrograph tends to support such a conclusion. Such structures have been called C, or contraction bands (22). In Fig. 9, they are grouped in isolated portions of the myofibrils with extreme shortening of the intervening sarcomeres. Such a configuration has been interpreted as a contraction node, possibly resulting from intense local contraction or injury (31).

Sarcoplasm. The sarcoplasm is readily distinguishable in the micrographs and appears to be the matrix in which lie the myofibrils, nuclei, and sarcosomes. There is no distinguishable membrane separating the myofibrils and the sarcoplasm; instead, the myofilaments seem to lie in a material which is continuous with the sarcoplasm. The sarcoplasm contains at least three major components: the sarcosomes, the sarcoplasmic reticulum, and the matrix. These three components have been described in electron micrographs of sectioned skeletal muscle by Bennett and Porter (5). The largest and most conspicuous of the components are the sarcosomes, which lie in great abundance between the myofibrils, with particular concentrations at sites of branching or junction of myofibrils and at the poles of the nuclei.

Frequently associated with the sarcosomes, but at times free from them, is a finely reticulated material, the sarcoplasmic reticulum, often vesicular in appearance (Fig. 4), which courses between the myofibrils, and which is usually most concentrated in the region of the Z band. Sometimes, as in Fig. 1, this reticulated material is seen to pass from the myofibril to the sarcolemma at the level of the Z. A much smaller concentration is occasionally seen in the region of the M band. However, sarcoplasmic reticulum in

cardiac muscle does not disclose the regularity of organization which is associated with it in skeletal muscle (5). A finely granular, more or less homogenous matrix is frequently found embedding the myofibrils and the sarcomeres. Unidentified sarcoplasmic inclusions are occasionally seen scattered among the sarcosomes. They appear as dense structures which are smaller than the sarcosomes and which differ from them in appearance and internal structure (Fig. 3).

Sarcosomes. The sarcosomes of cardiac muscle are almost as conspicuous as the myofibrils. They are round, oval, or filamentous in form, and lie between the myofibrils. Large clusters are found at the sites of anastomoses of adjacent myofibrils, and at the poles of the nuclei. Dense material or reticulum often bridges the space between sarcosomes and Z bands (Figs. 1 and 2). Although many sarcosomes are seen in close proximity to nuclei, no continuity between these two structures has been demonstrated.

Within the sarcosomes are numerous, regular, bi-walled structures lying in parallel array, usually with the long axis normal to the long axis of the myofibrils (Figs. 3 and 5). Each wall is approximately 200 Å thick with an intervening clear space of the same dimensions. The adjacent inclusions are separated one from another by about 600 Å. The number of these structures within any sarcosome is variable, ranging from 3 or 4 to as many as 40. They are usually enclosed within a thin walled membrane, the sarcosomal membrane, which separates them from the sarcoplasm. On cross section (Fig. 5), a few of these inclusions appear to be vesicular, suggesting a tubule-like structure cut across. At high magnifications a somewhat irregular periodicity of about 500 Å appears along the walls of these structures. Occasionally extremely fine strands cross from wall to wall at irregular intervals (A, Fig. 5). In addition to the above structures, the sarcosomes contain variable quantities of clear or finely granular material.

Internal sarcosomal structure of this sort have been observed in cardiac muscle tissue prepared both with and without phosphomolybdic and phosphotungstic acids, and after fixation with formalin as well as with osmium tetroxide. They resemble closely the structures described by Palade (22) in mitochondria of cells of various types, including the sarcosomes of skeletal and cardiac muscle of the rat. These structures, called by Palade "cristae mitochondriales", appear to be equivalent to the bi-laminar structures seen in mitochondria by Sjöstrand (30). Similar structures have been seen in mitochondria of many types of cells. Thus, as Palade (21) points out, a rather elaborate internal structure of this nature appears to be a very general characteristic of mitochondria, including sarcosomes.

Sarcolemma. Sarcolemma of cardiac muscle has been observed in both fragmented and sectioned material. The sarcolemmal fragments exhibit markings which are attributed to adherent sarcoplasmic reticulum. The horizontal stripes visible on the sarcolemma correspond generally in dimensions and distribution to the Z bands. In addition, irregular connections between these sarcolemmal markings have been seen which do not correspond to any configuration in the myofibrils. In sectioned heart muscle the sarcolemma is usually recognized as a fine structureless membrane (SL, Fig. 7) approximately 100 Å in thickness when measured in perpendicular cross section. In oblique sections (SL, Fig. 3), a projection of the sarcolemma in the plane of section may be 0.1 micra or more thick and may display a granular appearance, apparently due to adherent reticular material. Because of the uncertainties regarding the plane of section in relation to the sarcolemma, accurate measurements of this structure have not been obtained. On the outer surface of the membrane collagen fibrils are present in irregular array, recognized because of their characteristic 640 Å periodicity (24).

The myofibrils appear to be connected to the sarcolemma at irregular intervals by tenuous material connecting with the Z bands (A, Fig. 1). These connections are frequently absent or broken without apparent distortion of either the myofibrils or the sarcolemma. These Z band extensions are not distinguishable from sarcoplasmic reticulum.

Nucleus. The present material has contributed little to our knowledge concerning the structure of the nucleus. Its contents appear to be finely granular with occasional fine fibrous elements present. Frequently dense, irregular masses are seen, occasionally arranged in a rosette, which are considered to be nucleoli. The number of such structures is variable, apparently related to the plane of section, but usually 1 to 5 are seen. No structure has been noted which was interpreted as a chromatin network. The nucleus is enclosed by a membrane which is approximately 100 Å thick. Internal to the membrane, there is usually a more dense layer of nuclear material, which varies in thickness from 0.05 to 0.3 of a micron, and which rapidly dissipates itself inwardly without sharp delimitation from the remainder of the nuclear contents. Occasionally this layer appears to be composed of granules arranged in pallisading fashion around the circumference of the nucleus. In occasional nuclei numerous small vacuoles are seen in the nuclear wall.

Intercalated discs. These characteristic cardiac muscle structures were rarely encountered in this material. Structures resembling those described by Van Breeman (32) were occasionally seen.

DISCUSSION

An extensive literature has accumulated on cardiac muscle since Leeuwenhoek's first observations in 1694. Excellent reviews of the subject have been presented by Häggqvist (9) and Cohn (6). In recent years many have sought to transfer findings on skeletal muscle directly to cardiac muscle. Assumptions of close correspondence are probably valid to a certain extent, as is demonstrated in this study by the similarities of structure which skeletal and cardiac muscle have in common. However, one feature of cardiac muscle which sets it apart from skeletal muscle, is its comparative lack of regularity. The extensive branchings and anastomoses of fibers and myofibrils constitute one of the obvious differences. Another important difference is the relative obscurity of the striations in cardiac muscle.

Special interest and significance is attached to the variabilities found in the Z bands, particularly to their frequent tenuous appearance, as well as to their occasional complete absence. Speidel, studying living frog skeletal muscle, could observe no Z bands until the muscle was subjected to stimulation (31). A similar variability in the appearance of the Z bands has been observed in electron micrographs of sectioned skeletal muscle by Bennett and Porter (5). Whether all the changes in appearance of the Z bands are functions of muscle contraction cannot be stated at present.

It is further evident from this study that the concept of direct Z band continuity between adjacent myofibrils and to the sarcolemma is not correct for cardiac muscle. Such a concept has been advocated in recent years for skeletal muscle (10, 25, 7, 28), and for heart muscle by Beams *et al.* (3), and Kisch (14). It has recently been contested for skeletal muscle by Barer (2), who believes that continuity between adjacent Z bands is neither mechanically strong nor responsible for transmitting tension generated by the myofibrils during contraction. He attributes the alignment of sarcomeres in muscle to long range colloidal forces or to hydrogen bonds. Bennett and Porter's (5) studies on skeletal muscle and the present study on heart muscle lend support to Barer's contentions.

The dimensions of the myofilaments of cardiac muscle agree rather closely with similar measurements in skeletal and smooth muscle (7, 10, 28, 25). Following the isolation by von Muralto and Edsall in 1930 (33) of a muscle protein to which they applied the term, "myosin", subsequent extensive studies of this protein and the associated actin raised little doubt that this material was an important element in muscular contraction. The demonstration by Hall *et al.* (10), and many others of the resemblance of electron

micrographs of myosin or acto-myosin to the myofilaments of muscle have caused some to speak of the filaments as myosin "filaments" (10). The view is here taken, despite the attractiveness of such a concept, in which a tempting correlation between histology and biochemistry can be achieved, that there is no direct evidence that the myofilaments of cardiac muscle are identical chemically with any muscle protein such as myosin or actomyosin.

The sarcosomes undoubtedly play a very important role in the physiology of heart muscle. They were first described in striated muscle by Kölliker (17), who spoke of them as "interstitial granules". Regaud (26) later equated the sarcosomes of heart muscle to mitochondria. Watanabé and Williams (31) and Pappenheimer and Williams (23) studied the biochemical activity of insect flight muscle sarcosomes and were able to demonstrate that these inclusions are very highly active enzymatically and are probably closely related to, if not identical with, mitochondria. Recently, Harman and Feigelson (12) made further observations on heart muscle sarcosomes, using both biochemical and histological techniques, and came to the conclusion that they are indeed mitochondria. There is apparently a quantitative relationship of these sarcosomes to the relative activity of the muscle under study. Their number is great in highly active muscle such as vertebrate heart, insect flight muscle (34), and mammalian diaphragm, and comparatively low in some striated skeletal muscles (5), smooth muscle, and in the rudimentary insect heart muscle (23). Sarcosomes of vertebrate heart muscle are here shown to possess a detailed internal fine structure similar to that of mitochondria (22, 30).

The internal structure of the sarcosomes undoubtedly has considerable significance, possibly representing a framework on which are aligned enzymatically active protein molecules in such a way as to present maximum reactive surface to the substrates, with the active points arranged in orderly sequence. Palade (22) observed mitochondria in a wide variety of tissues of the rat, including the sarcosomes of heart muscle. His findings are very similar to the ones described above, but he interprets the internal mitochondrial structures to be internal ridges (*cristae mitochondriales*) protruding from the inner surface of the mitochondrial membrane. Sjöstrand (30) also described similar structures in the mitochondria of mouse kidney and retinal cells, and concluded that they represent double membranes, presumably of a protein character, separated by lipid molecules. He also regarded the external mitochondrial membrane as being a double membrane, but did not imply that the internal structures arise from the external membrane. In Figure 5 of this paper, the internal laminar and tubule-like struc-

TEXT TO FIGURES

Q, J, Z, H, M, E, N – cross bands of the myofibrils.

MF – myofibrils; Mf – myofilaments; S – sarcosomes; SM – sarcosomal membrane; ST – tubule- and lamella-like internal sarcosomal structures; SL – sarcolemma; SC – sarcolemmal collagen; SR – sarcoplasmic reticulum; Nu – nucleus; nuc – nucleolus; coll – collagen; C – contraction bands; CN – contraction nodes.

Magnification is indicated on each figure by a line representing one micron unless otherwise specified.

Fig. 1. Chicken ventricle fixed *in situ*, treated with .001 M phosphomolybdic acid. The arrangement of the myofibrils is typical for cardiac muscle, and demonstrates the numerous branchings and anastomoses which are characteristic for this muscle. The Z bands frequently show a lack of continuity between adjacent myofibrils. A general alignment of the myofibrils is well maintained. At A fine strands of sarcoplasmic reticulum join the Z to the sarcolemma.

Fig. 2. Frog ventricle, fixed *in situ*, treated with .001 M phosphotungstic acid. Sarcoplasmic reticulum is very prominent in this section, which shows particularly well the intervention of this material between adjacent Z bands and between Z and sarcosomes. Note the relative prominence of the H band and the absence of the J band.

Fig. 3. Chicken ventricle, fixed *in situ*, treated with .001 M phosphomolybdic acid. The branchings and anastomoses of the myofibrils are very prominent in this section, which also shows a considerable variability in the intensity of the Z bands, and a complete absence of other banding. The sarcolemmal collagen is seen to lie outside the myofibrils in the intermyofibrillar space. Sarcosomes are very prominent lying at a site of branching of the myofibrils. U is a dense unidentified organoid seen frequently, the nature of which is unknown.

Fig. 4. Chicken ventricle, fixed *in situ*, treated with .001 M phosphomolybdic acid. The myofilaments seem to be uniform along their entire course and are apparently continuous through the tenuous and finely granular Z bands. The clearing of the sarcomeres in the region of the H band is very subtle and does not appear to depend on any visible change in the configuration of the myofilaments. The E bands take a configuration of very fine granularity lying parallel to the Z bands. The wide band at A has been interpreted as an infolding of sarcolemma at this point. At B delicate interfilamentary bridging is observed. The very fine granules seen scattered throughout the myofilaments are of unknown significance. Vesicular configuration of sarcoplasmic reticulum is clearly evident in several areas.

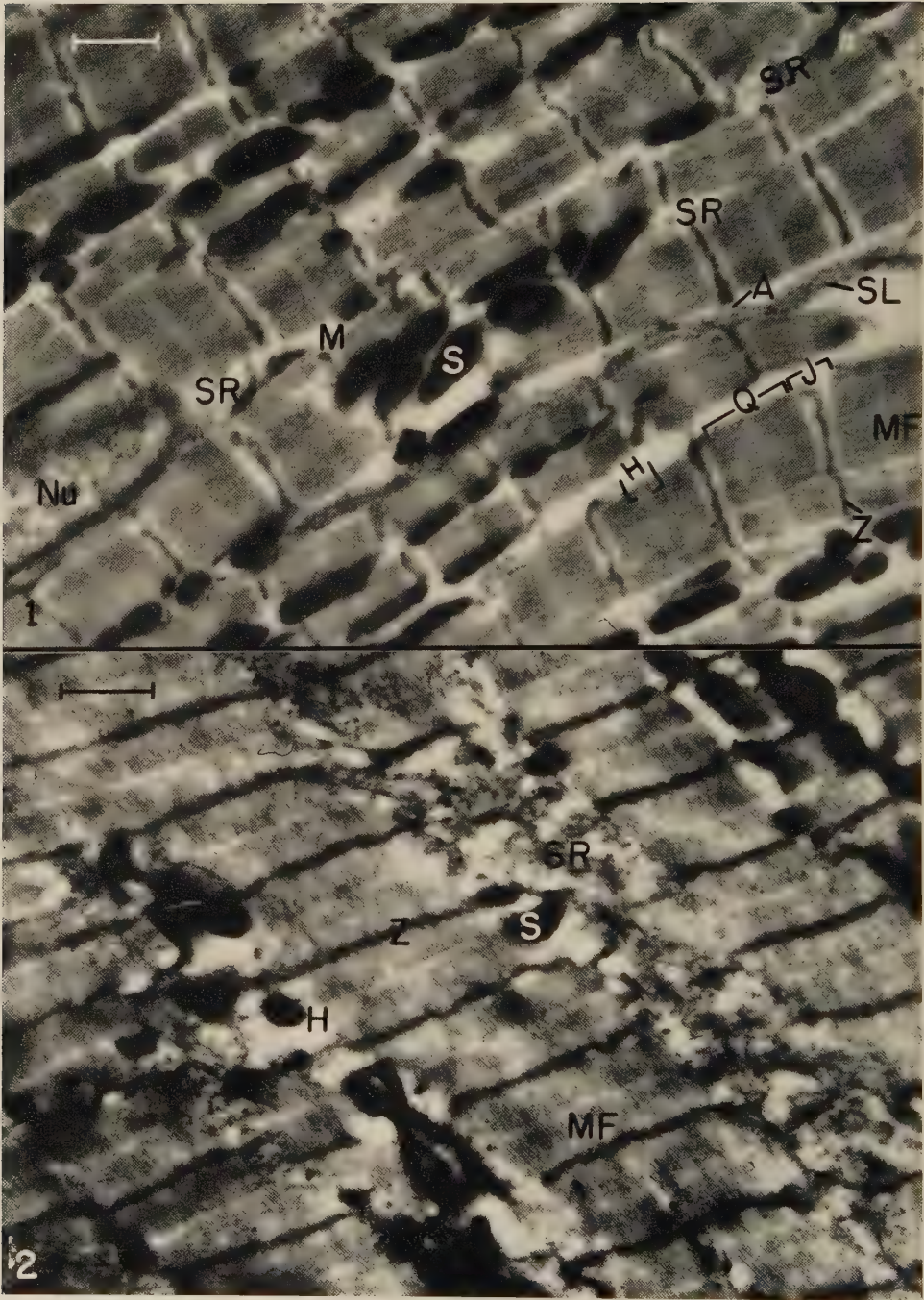
Fig. 5. Chicken ventricle, fixed *in situ*, treated with .001 M phosphomolybdic acid. Details of internal structure of the sarcosomes shown are: (A) the regular bi-walled laminar or tubule-like structures with fine strands crossing from wall to wall, (B) the cross sectional appearance of these structures appearing vesicular, and (C) the apparent lack of continuity between the internal structures and the sarcosomal membrane. At D fine interfilamentary bridges are present.

Fig. 6. Chicken ventricle, fixed *in situ*, treated with .001 M phosphotungstic acid. Almost complete absence of the Z bands in the presence of good alignment of the myofibrils and sarcomeres is evidence. At A a remnant of the Z band appears as an extremely fine granularity. Continuity of the myofilaments cannot be seen in the I bands. These two factors suggest that alignment of the myofibrils is dependent on factors other than the Z band.

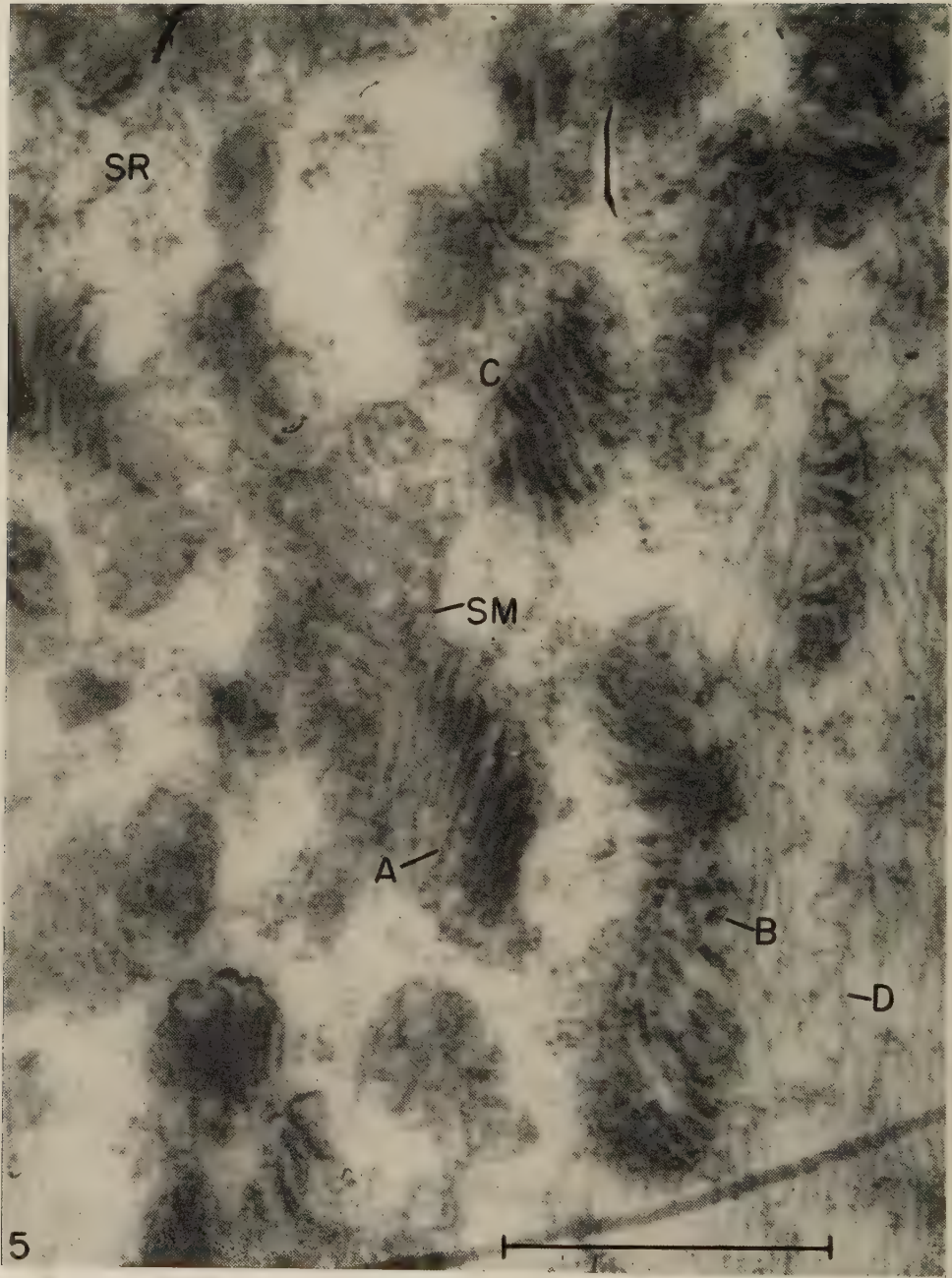
Fig. 7. Chicken papillary muscle, fixed *in situ*, treated with .001 M phosphomolybdic acid. The large bundle of collagenous fibrils lie in very close contact with the myofilaments, with no evidence of direct continuity between collagen and myofilament. At A the myofilaments are seen to be oriented towards the collagen fibrils. No membrane can be perceived in that area separating the two types of material.

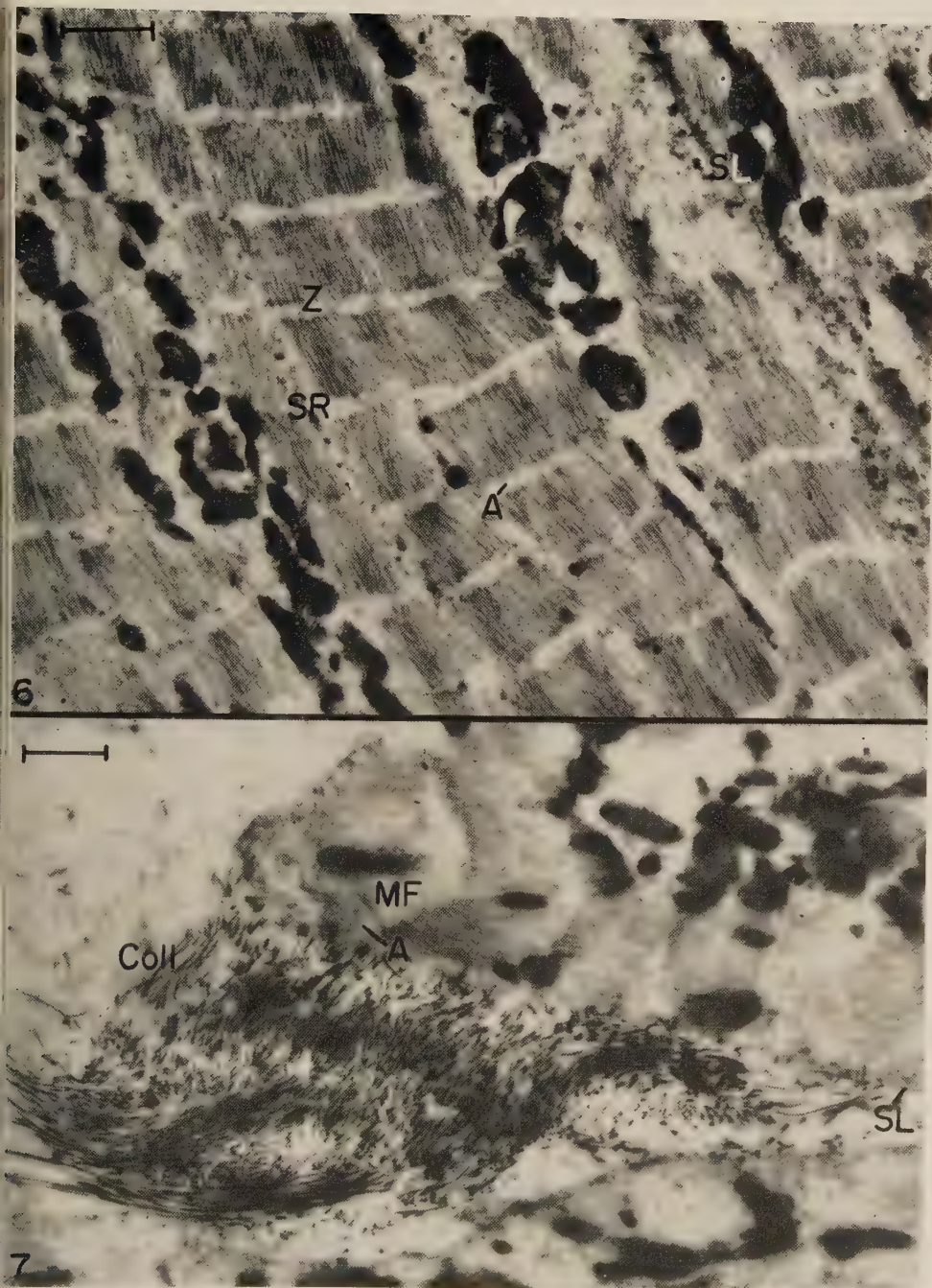
Fig. 8. Chicken ventricle, fixed *in situ*, treated with .001 M phosphotungstic acid. Portions of the myofibrils are in contraction with very dense contraction bands at the site of the Z. Some of the Z bands (A) appear to be double.

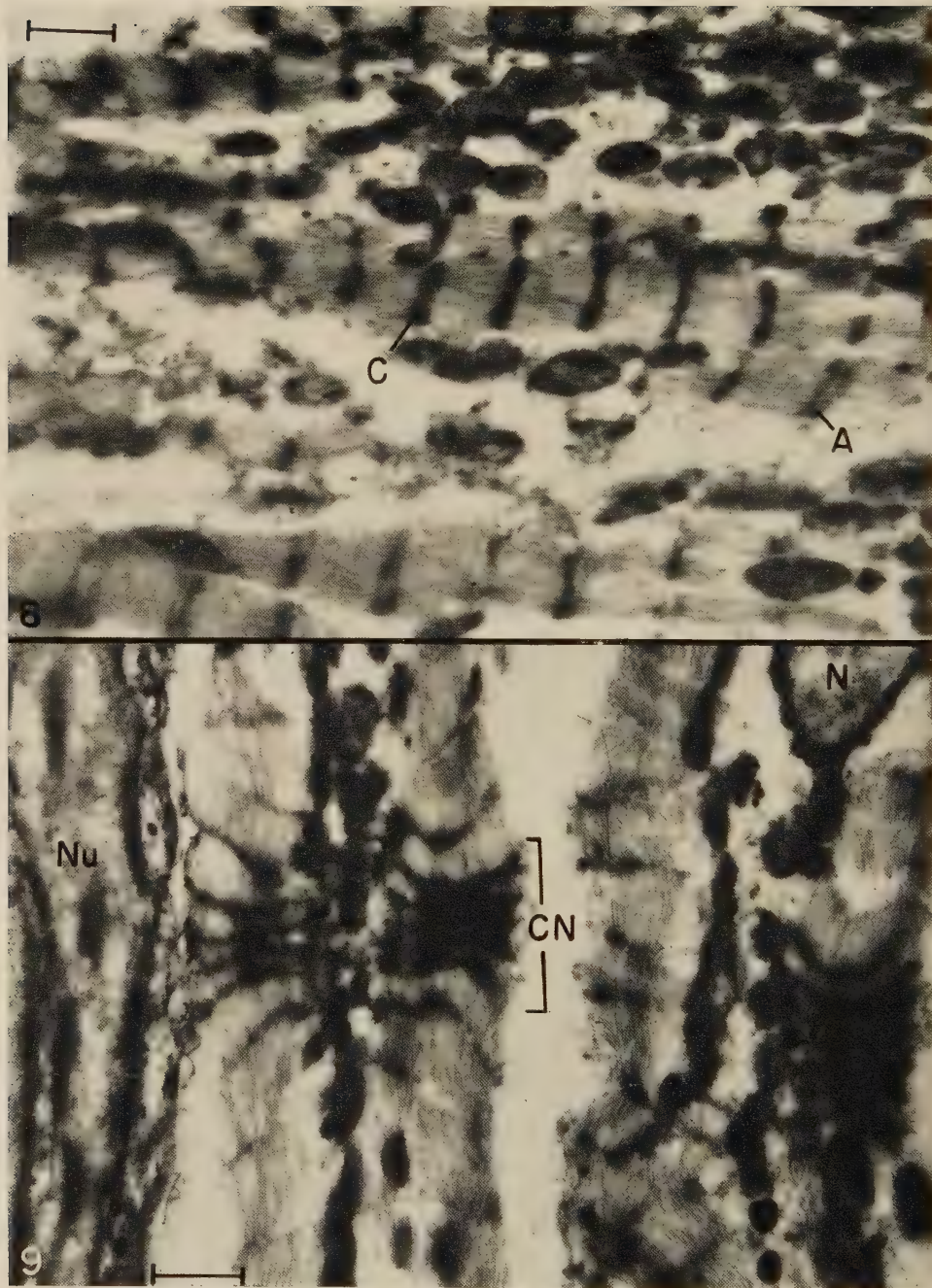
Fig. 9. Chicken ventricle, fixed *in situ*, treated with .001 M phosphotungstic acid. Three areas of very dense Z bands with extreme shortening of the intervening sarcomeres are interpreted as contraction nodes as described by Speidel (31).











tures do not, in general, appear to arise from or be connected to the sarcosomal membranes, and do not suggest a general validity for Palade's term, "*cristae mitochondriales*". The true geometry of these internal structures and their relation to the sarcosomal membrane remains to be clarified.

SUMMARY

1. Fragmented and sectioned cardiac muscle of chicken and of other vertebrates has been studied by electron microscopy. Some of the techniques employed in preparation are described.

2. Many features of the classical cytological features of cardiac muscle are confirmed, and close similarity of fine structure to that of skeletal muscle is established. An extensive branching and anastomoses of the myofibrils, corresponding in miniature to that of the fibers, has been demonstrated. A lack of regularity and a relative obscurity of the cross-stripes of the myofibrils have been found to distinguish electron micrographs of cardiac muscle from those of skeletal muscle.

3. Myofilaments are described as they appear in both fragmented and sectioned material. The diameter and periodic longitudinal arrangement of material along the myofilaments of fragmented muscle resemble those described in similarly prepared skeletal and smooth muscle. In sectioned material, the myofilaments show structures occurring approximately every 220 Å, forming bridge-like threads joining neighboring myofilaments, giving rise to a ladder-or lattice-like pattern.

4. Variabilities in the appearance of the various cross striations of the myofibrils are described, with particular emphasis on the Q, J, and Z bands. Evidence has been presented that the Z bands do not provide tension bearing connections between myofibril, nor between myofibril and sarcolemma.

5. Sarcosomes have been found in great numbers in cardiac muscle. Their relationship to mitochondria has been discussed. The internal structure present within the sarcosomes has been described in detail, and its significance suggested.

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AN IMPROVED ULTRAMICRO KJELDAHL TECHNIQUE¹

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FOR the determination of the amount of tissue used in biological and chemical work on an ultramicro scale, as for example in studies on the early embryo where the total amount of tissue available corresponds to approximately 0.5 to 5.0 μg protein nitrogen, no method has so far been devised which is superior to the conventional Kjeldahl technique. A number of good methods are available for determining total nitrogen in the range from 1 to 10 μg (2, 3), and recently Brüel, Holter, Linderstrøm-Lang, and Rozits (1) outlined a method for use in the range from 0.1 to 1 μg for which an accuracy of ca. 0.005 μg is claimed. There would be little justification for presenting a new technique were it not for the fact that the method herein described possesses certain features which make it superior, in many respects, to existing methods. The chief advantages of our method are: 1) relatively little specialized equipment is required, 2) the volumes of reagents are sufficiently large to make for ease in handling and to permit of duplicate or triplicate determinations on aliquots of the sample tested, 3) the time for digestion and distillation has been reduced so that a determination can be completed in 4 hours or less, and 4) the method is not only extremely sensitive but also extremely accurate.

The method as described below is similar in principle to the conventional macro or micro Kjeldahl technique, so that a detailed account of procedure need not be given. Suffice it to say that tissue or tissue homogenate is heated with a sulfuric acid digestion mixture, that aliquots of the diluted digest are neutralized with alkali in specially constructed vessels, and that the ammonia freed is then permitted to diffuse into an acid film of known volume and strength so that the amount of nitrogen in the sample can be determined titrimetrically.

Reagents

The digestion mixture is prepared by adding to 100 ml of 50 per cent H_2SO_4 (Mallinckrodt's low nitrogen analytical reagent) one gram each of CuSO_4 , K_2SO_4 , and SeO_2 .

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The receiving acid consists of 0.004 N H_2SO_4 , containing 4 ml of 0.4 per cent bromocresol green per 100 ml.

The standard alkali is 0.01 N NaOH. The solution is accurately standardized and protected from contamination and evaporation.

A color standard, to serve as a titration endpoint, consists of 4 ml of 0.4 per cent bromocresol green in 100 ml acetate buffer at pH 4.6.

In addition, ammonia-free water and saturated NaOH are required.

Digestion

Digestion is carried out in straight-walled 10 × 75 mm Pyrex brand ignition tubes (glass No. 172). The nitrogen source, either tissue or tissue homogenate, is delivered to the bottom of each tube by means of a calibrated constriction pipette after which exactly 24 μl of digestion mixture are added. Suitable blanks are similarly prepared.

The tubes are then pushed to a depth of about 2 cm into an electrically heated sandbath. They are supported in place by a perforated asbestos plate which by insulating the part of the digestion tube above it provides an effective but restricted zone of reflux. With this technique, even when the acid is made to fume strongly, no measurable loss of acid occurs.

The time necessary for complete digestion was found to be about three hours.

Diffusion

After digestion has been completed, the tubes are cooled and a given volume, usually 200 μl , of ammonia-free water is added to each. The contents are thoroughly mixed, and aliquots of known volume, about 90 μl , are then transferred to a series of diffusion vessels.

The diffusion vessel consists of a micro-florence flask whose dimensions are: inside diameter of bulb 10 mm, length of neck 22 mm, inside diameter of neck 5 mm. It can be easily constructed from tubing with a 5 mm bore. The neck of the diffusion vessel is coated with a thin film of paraffin wax (M.P. 72°C). Uniform pellets of wax are first prepared by dropping melted paraffin from a fine pipette onto a clean, cold glass plate. One pellet is placed in the neck, and the vessel is then maneuvered so that a rigidly-held heated silver wire can be inserted. The vessel is then rotated by hand with the result that melted paraffin is applied as a uniform coating along the entire length of the neck as far as the vessel bulb.

Next 36 μl of saturated NaOH solution, giving a final concentration of

about 4 N, are layered below the digest. In order to prevent contact of the alkali pipette with the vessel neck, a perforated glass thimble is first placed over the vessel mouth. The pipette can then be accurately guided by the edge of the perforation. Finally, by means of a constriction pipette a measured volume, about 45 μ l, of 0.004 N receiving acid is placed in the neck to form a column about 3 mm long. The acid column is positioned in the upper portion of the neck of the diffusion vessel so that following subsidence of the heat of neutralization, it does not move too far down the length of the flask neck. Initially, we used a lower concentration of acid since most of our nitrogen samples were in the range of 0.5 μ g or less. We found, however, that the rate of ammonia absorption could be increased appreciably by raising the acid concentration. This also extends the upper limit of the range of the technique.

The diffusion vessels are capped with small squares of Parafilm. After each film has been pressed in place, it is perforated with a fine needle to permit pressure equilibration during the diffusion process.

The diffusion vessels are then secured in a rotating stand. This stand consists of a thin aluminium disc to the rim of which 18 spring clips have been radially fastened. The clips are mounted so that the vessels are held at right angles to the plane of the disc. The effective diameter of the rotating stand is 25 cm.

The vessel stand is next mounted on a horizontal shaft in a constant temperature incubator, at 40°C, and rotated in a perpendicular plane at 30 rpm. Because of the rotation, the contents of the bulb are spread as a thin film over the bulb surface. Diffusion of the ammonia from bulb to acid is, therefore, very rapid and is complete in as little as one-half hour as compared to two hours required for stationary vessels.

Titration

Titration of the receiving acid in the diffusion vessel is done with standard 0.01 N NaOH delivered from a 10 μ l capacity Greiner microburette graduated in 0.01 μ l.

The diffusion vessel is held during the titration in a holder made from a segment of rubber tubing. The rubber holder is mounted on the tongue of an electric vibrating massager, and the amplitude of vibration is controlled by means of a Powerstat voltage regulator. The entire vibrator is fastened to a rack and pinion mount, so that the film can conveniently be brought into contact with the burette tip. The above arrangement provides excellent

stirring by vibration and eliminates the necessity of inserting anything, such as a magnetic flea or glass filament, into the thin film of acid being titrated.

A color standard is set up in another diffusion vessel, and the two films are illuminated by a narrow beam of light from a fluorescent source. Color matching with this arrangement is easily done, and the maximum end point error is of the order of 0.03 μl , corresponding to about 0.004 μg N (Table I).

TABLE I

Titration of 44.7 μl ca. 0.002 N H_2SO_4 with ca. 0.01 N NaOH
(brom cresol green as indicator).

Sample	μl NaOH	Deviation from mean
1	8.57	+0.02
2	8.55	0
3	8.55	0
4	8.53	-0.02
5	8.58	+0.03
6	8.52	-0.03
7	8.53	-0.02
8	8.53	-0.02
9	8.56	+0.01
10	8.55	0
Mean	8.55	

Standard deviation of mean ± 0.02 μl .

The nitrogen of the digested sample is obtained by multiplying the nitrogen content of the aliquot used for diffusion by a dilution factor. The total volume used in calculating the dilution factor represents the sum of the water added to the digestion flask at the completion of digestion and the volume of acid in the digestion mixture.

Tests of the method

The effective lower limit of the method was tested by determining the recovery of known amounts of nitrogen from ammonium sulfate solutions (Table II). The ammonium salt was treated as organic material and "digested" for 3 hours before dilution and transfer to the diffusion vessels. Recovery of nitrogen source from 0.15 μg up is complete in all cases.

TABLE II

Recovery of known amounts of nitrogen.

(NH ₄) ₂ SO ₄ (0.03 µg N/µl) as N source		
vol. sample µl	µg N	
	expected	obtained
5	0.150	0.150
10	0.300	0.298
20	0.600	0.593
40	1.200	1.200

In addition to the test with inorganic nitrogen, parallel determinations of the nitrogen content of a purified casein¹ preparation were made using the micro-Kjeldahl method of Parnas and Pregel and the present ultramicro technique. The difference in the amount of material used in the two methods is a hundred fold. Identical results were obtained with both techniques (Table III).

TABLE III

Comparison of nitrogen determination by micro (M) and ultramicro (ul-M) Kjeldahl.

N source	µl sample		µg N recovered/sample		µg N/µl sample	
	M	ul-M	M	ul-M	M	ul-M
Casein	1,000	10	216	2.16	0.216	0.216
Homogenate whole brain .	2,400	80	142	4.75	0.0592	0.0594
Optic lobe	1,150	11.5	44	0.436	0.0383	0.0379

Similar tests of the method were also made on homogenates of parts of tadpole brain. Appropriate aliquots of the same homogenates were used for the micro and ultramicro methods. Excellent agreement in the results obtained by the two methods is indicated in Table III. For casein as well as tissue homogenate a three hour period of digestion was found to be adequate for complete recovery.

¹ We are indebted to Dr. H. B. Vickery, Connecticut Agricultural Experiment Station, for a sample of casein of known nitrogen and moisture content.

SUMMARY

A modified ultramicro Kjeldahl technique has been described which is applicable to the determination of total nitrogen in the range from 0.1 μg and up with an accuracy of 0.005 μg .

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LES INCLUSIONS PROTÉIQUES DE L'OOCYTE DE *PARASCARIS EQUORUM*

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L'OOCYTE jeune de *Ascaris megalocephala*, de son vrai nom *Parascaris equorum* (Goerze 1782) est chargé de granules ribonucléiques (9, 10, 11, 12, 13). En cours de croissance ces granules ribonucléiques diminuent progressivement, tandis que deux sortes de corps figurés, décrits par van Beneden (1) sous les noms de «cristalloïdes» et de «sphères hyalines», et une importante quantité de glycogène à l'état diffus s'accumulent dans son cytoplasme (3).

Après la fécondation l'œuf s'entoure de deux membranes concentriques; l'externe, dite chitineuse, se forme aux dépens d'une partie du glycogène cytoplasmique; l'interne, constituée par une cire (alcool ascarylique de Flury (3)), se forme aux dépens des «cristalloïdes» de van Beneden (3). Entre temps, les «sphères hyalines» se rassemblent à la périphérie de l'œuf où elles semblent se fusionner avant de disparaître. L'ensemble de ces transformations se traduit par une forte diminution de la masse de l'œuf finalement isolé sous une faible couche de liquide périvitellin à l'intérieur de la membrane cireuse; l'œuf apparaît alors comme une sphère, protoplasmique ne contenant plus, en sus du chondriome et des pronuclei, que quelques globules gras et du glycogène diffus.

La membrane cireuse est imperméable à l'eau et aux solutions aqueuses, mais elle permet les échanges gazeux; on sait, depuis les recherches de Hallez, 1885, Samassa, 1898, Bataillon, 1910, etc.,¹ que la segmentation de l'œuf ne peut s'effectuer qu'en présence d'oxygène; mais le développement, jusqu'au stade de larve vermiforme mobile, s'effectue sans aucun apport de matériaux nutritifs, et l'embryon ne peut donc utiliser que ce qui reste inclus sous les membranes, principalement le glycogène ovulaire et la protéine constituant le «cône réfringent» du spermatozoïde. Panijel (9-11) et

¹ On trouvera la bibliographie des travaux anciens et récents relatifs à la physiologie et à la constitution chimique de l'œuf des Nématodes dans Fauré-Fremiet (3), Panijel (10) et V. Brand (2).

Pasteels (12, 13), ont démontré l'apparition d'acide ribonucléique dans la zone où le cône réfringent disparaît dans le cytoplasme ovulaire.

Le rôle et la signification des «sphères hyalines» restent indéterminés au cours des transformations qui accompagnent la fécondation et la segmentation. Leur nature même reste incertaine; l'un de nous (Fauré-Fremiet (3)) se fondant sur diverses réactions cytochimiques et chimiques¹ a conclu que les sphères hyalines sont des vacuoles aqueuses contenant un phosphate de calcium associé à des traces de protéines, et enveloppées par une couche limitante lipidique. Mais Wottge (15) ne retrouve pas au niveau de ces inclusions les réactions cytochimiques du Ca; Panijel (10) indique ces mêmes réactions comme incertaines, tandis que celle du phosphore selon Serra et Queiroz Lopes (14) lui apparaît positive, et que l'analyse chimique des sphères hyalines isolées en milieu alcalin lui montre une quantité importante de phosphore. Il conclut à «une réserve inorganique constituée par du P et du Ca, maintenue avec une structure d'élément figuré grâce à une pellicule limitante de nature lipidoprotéique».

Certains caractères de ces inclusions nous ayant paru peu compatibles avec une nature essentiellement minérale, nous avons repris l'étude des «sphères hyalines» de l'oocyte (4). Nous avons d'abord précisé leurs caractères de solubilité; la connaissance de ces derniers nous a permis de mettre au point des moyens de conservation corrects rendant possibles d'une part l'étude de l'évolution de ces inclusions et de l'autre celle de leur constitution par des techniques cytochimiques modernes; enfin nous avons mis au point les procédés d'isolement et de purification permettant d'aborder leur étude chimique.

Nous examinerons ici les résultats obtenus au point de vue cytologique et cytochimique; l'étude chimique fera l'objet d'un autre travail.

Aspect des sphères hyalines dans l'oocyte

Les différents stades de l'évolution des cellules sexuelles de *Parascaris equorum* se succèdent régulièrement tout au long du tube ovarien, depuis la zone germinative, où les oogonies sont en voie de multiplication, jusqu'à l'oviducte, où les oocytes mûres sont fécondés et forment leurs membranes, avant de passer dans l'utérus et dans le vagin.

¹ Aspect hétérogène montré par des colorants vitaux; insolubilité de la masse principale dans certaines solutions alcalines; réactions du P selon Macallum positive au niveau de la fraction insoluble; caractérisation chimique du P et du Ca dans cette même fraction isolée par centrifugation, mais non purifiée.

Les jeunes oocytes apparaissent comme des éléments piriformes disposés radiairement autour d'un cordon protoplasmique central ou rachis (Figs. 3 et 4); leur longueur s'accroît progressivement jusqu'à environ 200 μ , tandis que leur cytoplasme se charge d'inclusions deutoplasmiques ou vitellines (Fig. 5); au terme de leur croissance, ils se détachent du rachis sous forme de grosses cellules approximativement sphériques (Fig. 6).

L'examen des oocytes à l'état frais doit être fait dans le liquide coelomique, ou mieux dans une solution isotonique de saccharose. A cet effet, les tubes ovariens d'une femelle bien vivante sont rapidement extraits et lavés dans la solution de saccharose; de courts segments sont isolés à différents niveaux, et une légère pression exercée sur leur paroi en fait sortir un boudin d'oocytes que l'on dissocie rapidement dans une goutte de la solution sucrée pure ou additionnée d'un colorant vital.

Les globules hyalins apparaissent comme des inclusions sphériques, claires, faiblement réfringentes, séparées par un contour net du cytoplasme environnant qui est chargé de cristalloïdes très réfringents. Une légère pression exercée sur l'oocyte permet d'isoler ces sphères dans le liquide environnant, où elles conservent d'abord leur aspect caractéristique; mais dans ces conditions ces éléments paraissent relativement instables et se détruisent plus ou moins rapidement.

Le rouge neutre ne colore pas les sphères hyalines dans l'oocyte vivant; le bleu de méthylène et plus spécialement les bleus de Nil et de crésyl brillant les colorent au contraire d'une manière élective, avec une légère métachromasie; le vert Janus se fixe, lui aussi, sur ces éléments. Sous l'effet de ces divers colorants vitaux les sphères prennent rapidement un aspect hétérogène se traduisant par une vacuolisation et la séparation d'une masse corticale dessinant comme une calotte très fortement teintée en bleu ou en vert. La question se pose de savoir si les sphères hyalines sont réellement hétérogènes ou si les colorants vitaux produisent un effet de démixion de leurs constituants.

Après action d'un réactif fixateur assurant la conservation des sphères (voir plus loin), celles-ci sont électivement et intensément colorables par le bleu d'aniline utilisé suivant la technique de Mallory. Elles fixent d'autres colorants, tels que le picro-carmin (1), l'hématoxyline (6), etc., mais d'une manière beaucoup moins élective. C'est pourquoi nous avons utilisé systématiquement la triple coloration de Mallory pour nous assurer de la persistance ou de la disparition des sphères hyalines au cours des divers essais effectués pour déterminer leurs caractères de solubilité.

Caractères de solubilité des sphères hyalines

Les oocytes au stade piriforme, isolés dans une solution de saccharose isotonique, ont été traités dans une première opération pendant 12 heures par divers solvants et diverses solutions salines. Ils ont ensuite été examinés directement entre lame et lamelle, afin de contrôler la présence ou l'absence des sphères. Dans le cas où celles-ci étaient conservées, les oocytes ont été étalés en frottis et colorés selon la technique de Mallory. Dans un certain nombre de cas, cette seconde opération entraînait leur disparition. Il apparaît donc que les réactifs utilisés dans la première opération pouvaient produire soit une précipitation irréversible de la substance constituant les sphères hyalines, soit une précipitation réversible, rendant possible leur dissolution ultérieure lors de la coloration.

De ce fait on est conduit à distinguer 1) les réactifs qui insolubilisent définitivement les sphères hyalines, 2) ceux qui les conservent, mais sans les insolubiliser, et 3) ceux qui les dissolvent.

1) *Réactifs insolubilisants*. Rentrent dans cette catégorie: le bichlorure de mercure (HgCl_2) en solution saturée, qui donne de loin les meilleurs résultats, et l'acide phosphomolybdique en solution à 15 %.

2) *Réactifs conservant les sphères hyalines, mais sans les insolubiliser*. Les réactifs fixateurs de cette catégorie sont caractérisés par les deux tests suivants:

a) Action du réactif pendant 12 heures, étalement en frottis puis coloration selon la technique de Mallory: on constate la disparition des sphères hyalines.

b) Action de ces réactifs pendant 12 heures, étalement en frottis, traitement par HgCl_2 saturé, puis coloration selon la technique de Mallory: les sphères sont conservées. Le bichlorure de Hg a précipité de manière irréversible la substance des sphères hyalines, *conservée*, mais non *insolubilisée* par le réactif essayé.

Font partie de ce groupe de réactifs:

l'eau bouillante; l'alcool à 96° et l'acétone; les acides picrique à saturation et trichloracétique à 5 %; les solutions aqueuses des composés suivants: acétate de plomb à 10 %, sulfate d'ammonium à saturation et à demi-saturation, chlorure de sodium à 10 %, sulfocyanure de potassium à concentration supérieure à 2 M; formol à 10 %.

3) *Réactifs dissolvants*. La dissolution de la substance constitutive des sphères hyalines après traitement pendant 12 heures par ces réactifs à température ordinaire est prouvée par l'absence de coloration selon la technique de Mallory, même après l'action du bichlorure de mercure à saturation.

Rentrent dans cette catégorie:

l'eau distillée à froid; l'alcool à 50° et à 60°; les acides chlorhydrique, sulfurique, acétique, oxalique, métaphosphorique et sulfosalicylique, utilisées à toute concentration; les bases telles que soude, potasse, ammoniacque aux concentrations inférieures.

res à la normalité. (Au dessus de la concentration N, la soude et la potasse, tout en détruisant le cytoplasme, semblent laisser intactes les sphères hyalines. L'action insolubilisante de la potasse signalée par l'un de nous (3) et confirmée par Panijel (10) ne s'exerce donc qu'à partir d'une concentration déterminée (KOH N)); diverses solutions salines: chlorure de sodium entre 0.9 et 5 %; sulfate de cuivre à 10 %, iodure de potassium 3 M, sulfocyanure de potassium M; urée à 35 %.

Au point de vue technique nous retiendrons parmi tous ces caractères de solubilité des sphères hyalines:

- a) l'action insolubilisante irréversible de HgCl_2 ;
- b) la solubilité dans l'eau distillée;
- c) la solubilité dans l'alcool à 60°. (A des degrés alcooliques plus élevés, la dissolution devient de plus en plus difficile: les sphères hyalines sont insolubles dans l'alcool à 96°);
- d) la solubilité dans NaCl isotonique et à 5 % et l'insolubilité dans NaCl à 10 %;
- e) l'action du sulfocyanure de potassium: SCNK M dissout les sphères hyalines, mais à partir de la concentration 2 M, SCNK tout en dispersant totalement le cytoplasme, les laisse subsister isolées, un peu gonflées, souvent vacuolisées.

Ces caractères nous ont guidé d'une part dans le choix d'un fixateur permettant la conservation des sphères hyalines en vue de leur étude cytologique, d'autre part dans la mise au point d'une méthode d'isolement en vue de l'analyse biochimique de leur substance constitutive.

Étude cytochimique des sphères hyalines

1) *Choix du fixateur convenable.* Les caractères de solubilité précédents expliquent les résultats obtenus par divers fixateurs.

Les fixateurs de Carnoy et d'Allen ne conservent pas les sphères, en raison de la présence dans ces mélanges de substances solubilisantes. Il en est de même du sublimé acétique, en raison de la présence de l'acide. Les alcools à 80° et à 96° sont également à rejeter, puisqu'ils n'insolubilisent pas définitivement les sphères hyalines.

Le liquide de Bouin donne des résultats incertains: il conserve les sphères, sans doute grâce au formol, mais en les gonflant. Souvent on constate un déplacement de la substance constitutive à l'intérieur de la coupe.

On obtient par contre d'excellents résultats en remplaçant dans le liquide de Bouin, ainsi que le suggère Lams (6), l'acide acétique par l'acide trichloracétique (Bouin trichloracétique).

La composition du mélange de Helly: bichromate, sublimé, formol, explique également les bons résultats obtenus avec ce fixateur.

Enfin de magnifiques images sont obtenues en utilisant comme fixateur une solution aqueuse ou alcoolique saturée de sublimé. Le sublimé alco-

olique donne des résultats particulièrement intéressants dans la fixation des œufs pourvus de leur membrane cireuse.

L'emploi des derniers fixateurs (Bouin trichloracétique, Helly, solution aqueuse ou alcoolique saturée de sublimé) nous a permis d'aborder l'étude cytologique des sphères hyalines.

2) *Micro-incinération*. Avant d'indiquer les résultats des réactions cytologiques proprement dites, signalons ceux obtenus en soumettant des oocytes à la micro-incinération. Utilisant le four de Policard, nous avons incinéré soit des oocytes isolés dans le saccharose isotonique, soit des oocytes fixés par le Bouin trichloracétique. Dans le cas des oocytes isolés dans le saccharose isotonique, la quantité de cendres observée à la place occupée par les sphères hyalines est plus importante que celle donnée par les travées cytoplasmiques qui les entourent. Observées en fond noir, ces cendres se présentent comme un précipité très fin, amorphe, d'aspect homogène, diffusant une lumière bleuâtre (Fig. 1). On doit apporter une grande attention à la durée d'action du saccharose isotonique sur les oocytes. On constate en effet au cours du temps la disparition progressive du constituant minéral, mis en évidence par la micro-incinération, alors que le constituant organique, mis en évidence par les techniques cytologiques, persiste. La disparition du constituant minéral est encore plus marquée après action d'un fixateur acide comme le Bouin trichloracétique. Dans ce cas, la micro-incinération laisse la place des sphères hyalines absolument vide, alors que la coloration par la technique de Mallory montre la persistance du constituant organique (Fig. 2).

Nous avons essayé quelques réactions de caractérisation de ces cendres. Elles sont insolubles dans l'eau et difficilement solubles dans l'acide chlorhydrique dilué. La caractérisation du calcium par dépôt d'une micro-goutte d'acide sulfurique dilué en solution alcoolique (formation de cristaux de gypse) a donné des résultats négatifs ou très incertains.

La micro-incinération montre donc la présence simultanée, au niveau des sphères hyalines, de constituants organiques et minéraux. Les réactions cytologiques effectuées sur des oocytes totaux ou sur des coupes d'oocytes ne nous ont au contraire permis de caractériser avec certitude que le constituant organique.

3) *Réactions du phosphore*. Les travaux antérieurs sur la constitution des sphères hyalines ayant fait admettre la présence de phosphore, nous nous sommes particulièrement attachés à la recherche cytologique de cet élément.

Nous avons d'abord recherché la présence de phosphates minéraux par la réaction au nitrate de plomb, suivie d'un traitement par le sulfure d'am-

monium (réaction de Gomori sans incubation enzymatique). Cette réaction, quoique non spécifique, aurait en effet donné une indication intéressante, si elle avait été positive. Or elle nous a donné un résultat négatif.

Nous avons ensuite appliqué deux méthodes de détection du P organique: la méthode d'Angeli et celle de Serra et Queiroz Lopes (14). Toutes deux ont été systématiquement négatives.

Rappelons que l'un de nous (Fauré-Fremiet (3)) avait trouvé la réaction du P selon Macallum positive (action du molybdate suivi de phénylhydrazine). Or on sait aujourd'hui que cette technique est défectueuse (7), la coloration verdâtre obtenue étant souvent causée par une absorption du molybdate sur diverses structures (en particulier protéiques), et la phénylhydrazine étant capable de réduire en bleu les molybdates tout comme les phosphomolybdates. Le résultat positif obtenu sur les sphères hyalines n'avait donc pas de valeur.

Le résultat positif obtenu récemment par Panijel (10) avec la technique de Serra et Queiroz Lopes nous semble par contre beaucoup plus surprenant. Nous n'avons pu le reproduire et cette divergence nous paraît inexplicable.

4) *Réactions du calcium*. Les réactions cytochimiques du calcium nous ont donné des résultats contradictoires. Alors que la réaction argentique de von Kossa est négative, la réaction à l'alizarine de Crétin nous est apparue faiblement positive au niveau des sphères hyalines; Wottge (15) avait obtenu avec cette dernière des résultats négatifs et Panijel (10) des résultats incertains.

5) *Réactions des lipides*. Les réactions des lipides (Soudan III, bleu BZL) ont été négatives.¹

6) *Réactions des polysaccharides*. La réaction de Hotchkiss, particulièrement intense dans le cytoplasme environnant par suite de la présence de glycogène, est négative au niveau des sphères hyalines.

7) *Réactions des protéines*. Diverses réactions des protéines sont positives: réaction xanthoprotéique, réaction de Millon-Bensley, réaction de Thomas (ces deux dernières faiblement). Le caractère protéique du constituant des sphères hyalines est confirmé par le résultat positif obtenu avec la récente méthode de Mazia, Brewer et Alfert (8) au bleu de bromophénol mercurique, réaction qui semble spécifique des protéines.

8) *Conclusions de l'étude cytochimique*. Les réactions précédentes conduisent à admettre une nature protéique de la substance constituant les sphères

¹ La couche superficielle observable lors des colorations vitales a été considérée par l'un de nous (Fauré-Fremiet (3)) et par Panijel (10) comme de nature lipidique ou lipoprotéique. Cette hypothèse, fondée sur des caractères peu significatifs, ne nous paraît pas devoir être retenue.

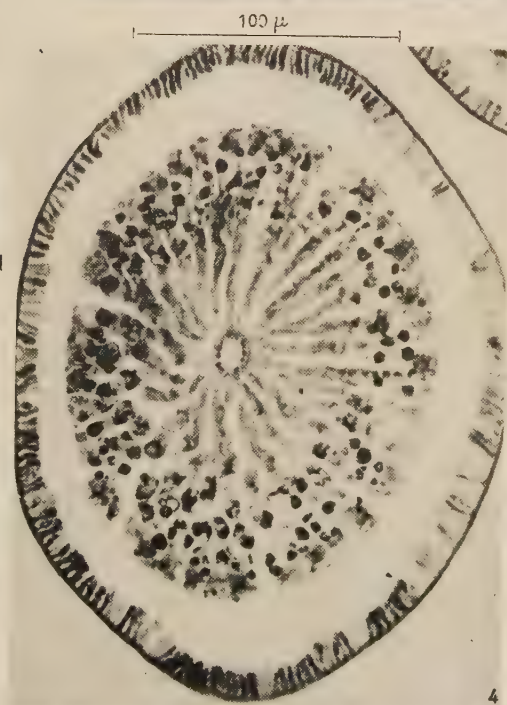
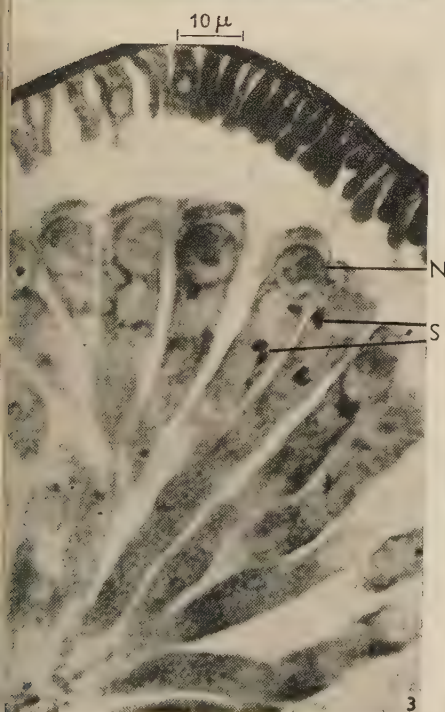
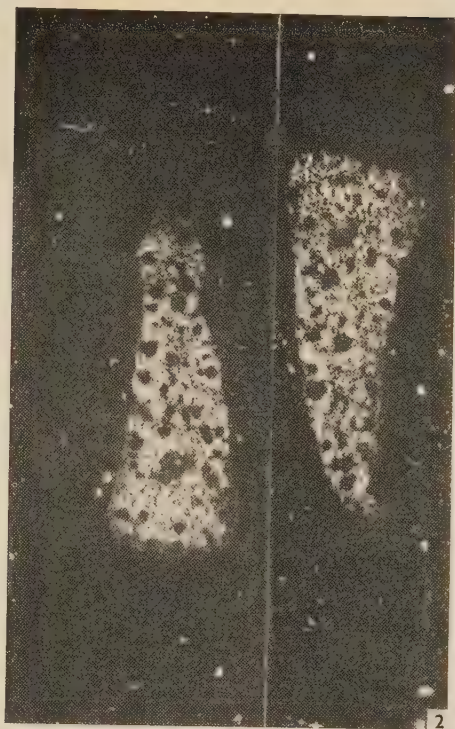
hyalines. Ce résultat s'accorde d'ailleurs avec la disparition par la pepsine et la trypsine signalée par Panijel (10). Il ne s'agit pas d'une nucléoprotéine (réaction de Unna et de Feulgen négatives), ni d'une protéine soufrée (réaction au plombite de sodium et réaction des groupements SH de Chèvremont négatives).

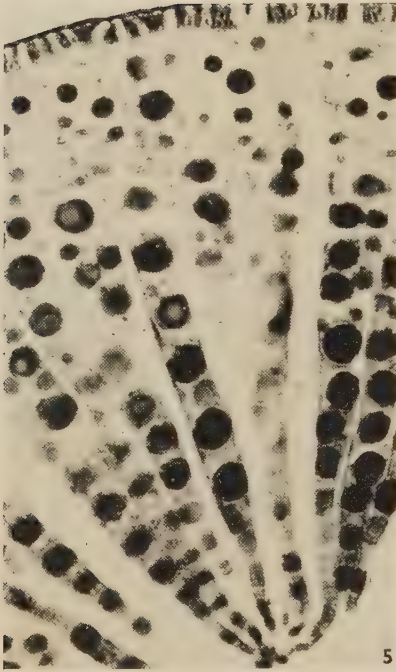
Les résultats de la micro-incinération montrent cependant que cette protéine n'est pas l'unique constituant des sphères hyalines. Elles renferment également des éléments minéraux. Il ne semble pas que ces derniers soient solidement liés à la protéine, puisque l'action de la solution de Bouin trichloracétique ou même l'action un peu prolongée du saccharose isotonique élimine facilement les éléments minéraux, laissant au contraire la protéine en place. Nous ne pouvons nous prononcer sur la nature de ces substances minérales, les diverses réactions essayées (en particulier celles du phosphore et du calcium) étant négatives ou incertaines. On peut penser que la protéine constituant les sphères hyalines se trouve dissoute ou dispersée dans une solution de sels minéraux.

Étude biochimique

L'étude biochimique devant faire l'objet d'un autre travail, nous indiquerons seulement ici les principaux résultats obtenus à cet égard. Une technique permettant l'isolement et la purification de la substance des sphères hyalines a été mise au point en tenant compte de sa solubilité dans NaCl isotonique et dans l'alcool à 60° et de son insolubilité dans NaCl à 10 % et dans SCNK 3 M. Après dialyse, l'analyse de la substance obtenue a donné une teneur en azote de 16,2 % et n'a révélé que des traces de phosphore (0,2 %). Le produit donne les réactions des protéines (réactions xanthoprotéique, du biuret, de Millon positives). Les réactions des nucléoprotéines sont négatives. La réaction au plombite de sodium et les réactions des glucides (réaction de Molisch, réaction de Dische à l' α -naphtol et à la di-phénylamine) sont négatives.

-
- Fig. 1. Spodogramme d'oocytes piriformes isolés dans une solution de saccharose isotonique; microphotographie en fond noir; les sphères hyalines apparaissent comme des masses blanchâtres.
Fig. 2. Spodogramme d'oocytes piriformes fixés par la solution de Bouin trichloracétique; microphotographie en fond noir; les sphères hyalines sont marquées par des espaces vides.
Fig. 3. Jeunes oocytes piriformes montrant l'apparition des premières sphères hyalines dans la zone apicale au-dessous du noyau (Bouin trichloracétique, coloration de Mallory). N, noyau, S, sphères hyalines.
Fig. 4. Oocytes piriformes plus avancés, montrant l'augmentation du nombre des sphères hyalines, fortement colorées, encore localisées dans la région apicale (Bouin trichloracétique, coloration de Mallory).





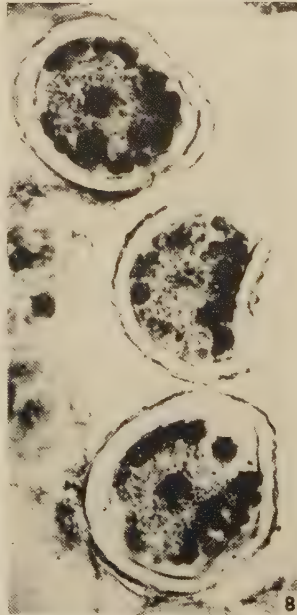
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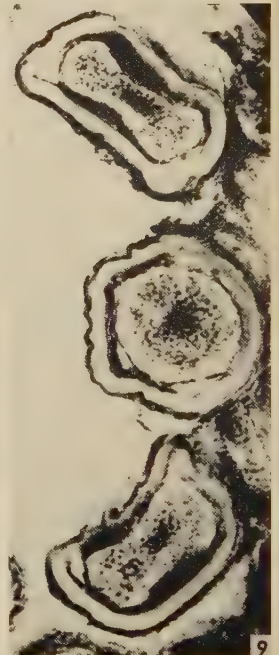
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L'analyse par chromatographie sur papier après hydrolyse a mis en évidence un certain nombre d'acides aminés, parmi lesquels une proportion relativement importante de proline.

L'électrophorèse à pH 8,6 et à pH 3,5 montre une mobilité assez faible et la présence d'au moins deux pics dans le diagramme, témoins de l'hétérogénéité de la protéine.

L'étude chimique confirme donc les résultats de l'étude cytochimique: la substance des sphères hyalines est constituée par une protéine électrophorétiquement non homogène, dont il convient, dès maintenant, de signaler les propriétés inusuelles de solubilité et la composition particulière.

Évolution des sphères hyalines au cours de l'accroissement de l'oocyte et de la maturation de l'œuf fécondé

La coloration de Mallory après fixation au liquide de Bouin trichloracétique ou au sublimé alcoolique a permis de suivre, sur les coupes pratiquées à différents niveaux de l'ovaire et de l'utérus, les stades successifs de l'évolution des sphères hyalines depuis leur apparition dans l'oocyte jeune jusqu'à leur disparition dans l'œuf fécondé.

C'est dans les jeunes oocytes piriformes mesurant environ 60 à 70 μ de long que les sphères hyalines commencent à apparaître sous forme d'inclusions peu nombreuses, irrégulières, intensément colorées par le bleu d'aniline et mesurant jusqu'à 5 à 6 μ environ (Fig. 3). D'abord localisées dans la région moyenne de l'oocyte, au-dessous du noyau, ces inclusions deviennent ensuite de plus en plus nombreuses (Fig. 4); chez les grands oocytes mesurant 200 à 300 μ , elles envahissent toute la masse cytoplasmique, sous forme de sphérules atteignant jusqu'à 10 à 15 μ de diamètre, les plus petites étant particulièrement nombreuses dans la région périnucléaire (Fig. 5).

Lorsque, arrivée au terme de leur croissance, les oocytes se détachent du rachis et prennent une forme sphérique, les inclusions sont distribuées

Fig. 5. Oocytes piriformes au terme de leur croissance (Bouin trichloracétique, coloration de Mallory).

Fig. 6. Oocytes mûrs, libres, ayant pris la forme sphérique (Bouin trichloracétique, coloration de Mallory).

Fig. 7. Œufs fécondés, montrant la margination des sphères hyalines (Bouin trichloracétique, coloration de Mallory).

Fig. 8. Formation de la membrane chitineuse et début de coalescence des sphères hyalines (Bouin trichloracétique, coloration de Mallory).

Fig. 9. Coalescence et début de disparition des sphères hyalines (Bouin trichloracétique, coloration de Mallory).

au hasard dans leur cytoplasme (Fig. 6); c'est à ce stade qu'a lieu la fécondation.

Après la pénétration du spermatozoïde, les sphères hyalines sont repoussées à la périphérie de l'œuf (Fig. 7), tandis que les cristalloïdes se rassemblent dans la partie centrale; cette «margination» bien connue des anciens auteurs a fait penser (5, 6) que les inclusions hyalines participent à la formation de la première membrane; nous ne retiendrons pas cette hypothèse, car les transformations ultérieures des sphères protéiques semblent tout à fait indépendantes de l'épaississement rapide de la membrane chitineuse.

Lorsque la première membrane a atteint son épaisseur maxima, les sphères hyalines deviennent coalescentes (Fig. 8) et semblent confluer en plages marginales irrégulières pour dessiner finalement un liseré plus ou moins continu autour de l'œuf (Fig. 9). C'est à ce moment que les cristalloïdes disparaissent et que la membrane cireuse se forme entre la membrane chitineuse externe et la masse protoplasmique, notablement réduite, de l'œuf. A partir de ce stade, le liquide de Bouin trichloracétique ne pénètre plus et une fixation correcte de la substance protéique des sphères hyalines ne peut être obtenue que par le sublimé alcoolique; il apparaît alors que la couche superficielle formée par la substance protéique se disloque, prend un aspect réticulé, puis disparaît dans l'étroit espace périvitellin compris entre la masse protoplasmique de l'œuf et les deux membranes qui l'enveloppent. Cette dernière transformation est probablement de courte durée, si l'on en juge d'après la zone très étroitement limitée de l'oviducte où l'on peut en observer les aspects caractéristiques.

La margination des sphères hyalines, suivie de leur confluence, de leur extension, et enfin de la disparition apparente de leur substance, paraît indiquer une modification de leur constituant protéique, dont un autre aspect est donné par la variation progressive de leur indice de réfraction.

Examinant les oocytes à l'état frais en utilisant soit la méthode des franges de Becke en lumière polarisée, soit le contraste de phase et plus spécialement le dispositif «varicolor» de Locquin, on constate que, avant la fécondation, l'indice de réfraction des sphères hyalines est nettement supérieur à celui du cytoplasme qui les entoure, tandis qu'à partir du moment où elles commencent à confluer, leur indice diminue, jusqu'à être sensiblement égal à celui du cytoplasme, puis devient notablement plus faible que celui-ci.¹

¹ Comme il s'agit ici d'un indice relatif, il serait intéressant de savoir si l'indice de réfraction du cytoplasme varie lui aussi et dans quel sens; il ne semble pas que cet indice augmente et la disparition d'une quantité importante de son contenu en glycogène, au cours de la formation de la première membrane, laisse plutôt supposer qu'il doit diminuer lui-même; s'il en est bien ainsi, la diminution de l'indice relatif des sphères hyalines garde toute sa signification.

La fonte des sphères hyalines, comme le changement des propriétés physiques qui la précède, suggère l'idée d'une dégradation de leur constituant protéique aboutissant à la dissolution de polypeptides, ou peut être d'acides aminés, dans le liquide périvitellin au sein duquel l'embryon se développe ultérieurement.

DISCUSSION

Les résultats que nous venons d'exposer infirment totalement les conclusions avancées quarante ans plus tôt par l'un de nous (Fauré-Fremiet (3)) et encore admises plus récemment (Panijel (10)), à savoir que les inclusions protoplasmiques de l'oocyte de *Parascaris equorum* décrites sous le nom de «sphères hyalines» sont de nature minérale et principalement constituées par un phosphate de calcium. Nos nouvelles recherches montrent en effet que ces inclusions sont essentiellement constituées par une protéine, dont les caractères très particuliers de solubilité permettent la caractérisation cytochimique *in situ*, ainsi que la séparation, la purification et l'étude chimique *in vitro*.

Devant ces faits nouveaux nous devons examiner deux questions relatives l'une à la constitution physicochimique de ces inclusions, l'autre à leur rôle physiologique.

L'examen des oocytes à l'état frais dans un liquide physiologique en présence d'un colorant vital tel que le bleu de Nil montre le plus souvent les sphères hyalines entourées d'une pellicule plus fortement colorable, apparemment visqueuse, et pouvant se condenser latéralement en formant une ou deux calottes plus réfringentes que la partie centrale de la sphère; après fixation, on observe très fréquemment soit le même aspect, soit une vacuolisation plus ou moins accentuée des sphères hyalines. Ces observations ont conduit à considérer les sphères hyalines de van Beneden comme des systèmes complexes, structurés, entourés par une pellicule de nature lipidique (Fauré-Fremiet (3)) ou lipoprotéique (Panijel (10)), d'après l'action des détergents et des ferments protéolytiques).

S'il en était bien ainsi, la protéine que nous avons isolée ne représenterait que l'un des constituants des sphères hyalines; mais il semble qu'une autre interprétation soit valable.

Si, utilisant une étuve à microscope, on examine des oocytes fraîchement isolés dans une solution de saccharose isotonique additionnée de bleu de Nil et maintenue à la température de 37 à 38°C, on constate que les sphères hyalines restent parfaitement homogènes, au contraire de ce que l'on observe à la température du laboratoire. Considérant d'autre part les aspects

plus ou moins fortement vacuolisés montrés par les sphères isolées dans une solution saline et tout spécialement dans les solutions concentrées de sulfo-cyanure de potassium, il est permis d'expliquer l'apparente hétérogénéité de ces inclusions cellulaires par un effet de démixion, lié à un gonflement traduisant un début de dissolution de leur contenu protéique.¹

L'homogénéité optique, à l'état normal (température de 37°C), des sphères hyalines permet d'admettre que les procédés d'extraction utilisés intéressent la totalité de leurs constituants organiques; cette conclusion ne contredit pas, bien entendu, la coexistence probable de plusieurs constituants protéiques de propriétés très voisines indiquée par les premiers résultats de l'électrophorèse, ni la présence d'éléments minéraux, mise en évidence par la micro-incinération, dans le liquide de dispersion de ces protéines.

La nature protéique des sphères hyalines motive, en tant que fait nouveau, quelques commentaires. En effet, la composition essentiellement minérale qui leur était assignée par les travaux précédemment cités conférait à l'oocyte de *Parascaris* un caractère assez exceptionnel² qui disparaît aujourd'hui. De même la démonstration de leur constitution protéique fait tomber l'intéressante opposition tracée par Panijel ((10), p. 100) entre l'ovogénèse de *Rana fusca* qui « met en jeu un métabolisme de synthèse et d'accumulation, et en particulier de synthèse et d'accumulation protéique », et l'ovogénèse du *Parascaris equorum* montrant surtout « un métabolisme de transformation et, en particulier, un métabolisme non protéique ».

En fait, dans l'un comme dans l'autre cas, la croissance de l'oocyte s'accompagne de l'élaboration d'un matériel protéique mis en réserve sous forme d'inclusions cytoplasmiques, de globules vitellins en un mot. Et la diminution du taux des ribonucléoprotéines cytoplasmiques, bien mise en évidence par Panijel au début de l'apparition des sphères hyalines, ne fait plus exception à la règle puisqu'elle correspond, ici comme dans tant d'autres cas, à la synthèse de composés protéiques.

On remarquera encore que ces constatations restreignent la signification accordée par quelques auteurs à l'apport, au cours de la fécondation de l'œuf chez *Parascaris*, d'inclusions protéiques spermatiques (ascaridine du cône réfringent); ces inclusions ont été comparées par Hertwig, par Scheben, par Marcus³ à un vitellus, et par Panijel ((10), p. 264), d'une manière plus

¹ Une figure publiée par l'un de nous (E. Fauré-Fremiet (3) pl. XII, Fig. 4) et reproduite par Panijel ((10) Fig. 10, p. 83) représente certainement un stade initial de la dissolution de deux sphères hyalines isolées dans le serum physiologique.

² Panijel (1) écrit (p. 83): « un tel cas de « réserve minérale » est à notre connaissance le seul rencontré jusqu'à présent dans les diverses ovogénèses étudiées ».

³ Voir pour la bibliographie de ces anciens travaux E. Fauré-Fremiet (3).

suggestive, à un «vitellus exogène», capable de suppléer à l'incapacité protéinogène de l'oocyte. Cette remarque ne touche d'ailleurs en rien l'intérêt tout particulier que présente la «recharge» en ribonucléoprotéines de l'œuf de *Parascaris* découverte par Panijel et Pasteels (9-13), recharge qui semble liée aux transformations subies par la protéine spermatique.

On n'oubliera pas, cependant, le fait bien particulier que le vitellus protéique de l'œuf de *Parascaris* est expulsé du cytoplasme peu après l'insémination et qu'il disparaît dans le liquide périovulaire; il resterait donc à vérifier la présence dans ce liquide de produits de dégradation solubles et à savoir si, au cours de la segmentation et de l'embryogénèse, ces produits sont utilisés par les blastomères et les cellules en voie de division.

RÉSUMÉ

L'étude cytochimique des inclusions contenues dans l'oocyte de *Parascaris equorum* et connues sous le nom de «sphères hyalines» de van Beneden montre qu'elles ne sont pas de nature essentiellement minérale, comme l'affirmaient les travaux antérieurs, mais qu'elles sont principalement constituées par une protéine possédant certains caractères particuliers: solubilité dans l'alcool à 60°, l'eau, le sérum physiologique et les acides; insolubilité dans le chlorure de sodium à 10 %, dans le sulfocyanure 2 M et dans les solutions alcalines concentrées. L'analyse chimique de cette protéine fait apparaître un taux de proline assez élevé.

L'étude de l'évolution de ces sphères au cours de la croissance de l'oocyte et de la maturation de l'œuf fécondé montre que, d'abord réparties au hasard dans le cytoplasme de l'oocyte, elles passent à la périphérie de l'œuf après fécondation, deviennent coalescentes et disparaissent dans l'espace périovulaire compris entre la masse protoplasmique et les membranes. L'évolution de ce vitellus protéique et son apparente disparition après la fécondation suggèrent sa transformation en produits de dégradation utilisables au cours du développement embryonnaire.

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VERSUCHE ZUR ANALYSE DES Ag-DICHOISMUS GLATTER MUSKELN KULTIVIERT *IN VITRO*

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TROTZ vieler Bemühungen kann das Wesen *dichroitischer Versilberungen* in der Histologie auch heute noch nicht als geklärt betrachtet werden. Die Deutung des Ag-Dichroismus i. S. von Ambronn als gerichtete Einlagerung geeigneter Leptonen des Metalls bleibt wegen gelegentlichen Ausbleibens des Dichroismus trotz reichlicher Metallsalzaufnahme in manchen Fällen unsicher (14), so dass auf besondere allotrope Molekelform und damit wirksam werdende Nebenvalenzen der Hydratgruppen des Gewebesubstrats geschlossen worden ist (14). Nach Ermittlungen von Frey-Wyssling und Wälehli (3) scheint es allerdings einer besonderen anisotropen Modifikation des Ag auch in der Spektralregion der mittleren und langen Wellen nicht zu bedürfen; mindestens sind die Differenzen zwischen gemessenen und aus der Anwendung der Wienerschen Formeln der Mischkörper errechneten Dichroismus-Werte „not of such a nature that it is necessary to assume an unknown allotropic modification of silver with particular optically anisotropic properties“. So sei hier kurz über Versuche, die zur Klärung der Frage angestellt worden sind, berichtet. Wegen ihrer ausgezeichneten Eignung zu polarisationsoptischen Messungen (13) sind dafür *Kulturen in vitro*, und zwar von *glatten Muskeln* gewählt und die Untersuchungen mittels Phasen- und Polarisationsmikroskopie und mittels Messungen der Doppelbrechung (Δn), und, nach Ag-Imprägnierung, des Dichroismus (Δk) ausgeführt worden.

MATERIAL UND TECHNIK

Zur Untersuchung dienten aseptisch dem Parapodiumrande bei *Helix pomatia* entnommene glatte *Muskeln*, die kurz in 0,4 % Chloraminlösung in isotonischem Ringer gespült und in mehrfach gewechseltem sterilem Ringer bis zum Verschwinden des Cl-Geruchs der Waschflüssigkeit gewaschen wurden. Die *Kulturtechnik* wird zweckmässig den Vorschriften von Gatenby, Hill und MacDougal (10) angeglichen, nur sollten unbedingt Cystin und nach Erfahrungen von A. Fischer, Astrup, Ehrensvärd und Oehlenschläger (4) Glutaminsäure als Aminosäuren dem Medium zuge-

setzt werden. Um gelegentliche *Dehnungsversuche* mit den Kulturen durchzuführen, sind diese in solchen Fällen statt auf gläsernen Objektträgern auf geeignet geformten Cellophanblättchen aufgepflanzt und mikrurgisch mit diesen gedehnt worden.

Zur *Versilberung* der explantierten Gewebe ist wiederum statt des von Schmidt (15) vorgeschlagenen S. Ramón y Cajalschen Verfahren die Imprägnationsmethode nach Weber (20) in der Modifikation nach Baud (1) mit viertägiger Vorbehandlung mit Mischung aus aq. dest. 94, Pyridin 4 und Formol 2 cm³ und mittels 3 % wässriger AgNO₃-Lösung angewandt worden. Teilweise sind auch partielle Ag-Imprägnationen explantierter Kulturen vorgenommen worden, indem ein bestimmter Anteil der Präparate mittels aufgeklebter Cellophanblättchen abgedeckt wurde.

Für die *phasenmikroskopische* Beobachtung ist mit dem 16 mm Objektiv die 0,2 A + 0,33 λ Beugungsplatte und mit dem 4 mm Objektiv der niedrigere und mittlere A $\pm$ Kontrast und niedrigere B-Kontrast, bei Ölimmersion der starkleuchtende Kontrast 0,07 A + 0,25 λ zu empfehlen (vgl. auch (8, 19)). Die Kombination von Prinzipien der Phasenmikroskopie und der Polarisationsoptik zur Variation der Phasendifferenz von $-\pi$ bis $+\pi$ und zugleich des Amplitudenverhältnisses von 0 bis ∞ ist nach Arbeitsvorschriften von Österberg (9) vorgenommen worden (12).

Die *Messung der Δn* wurde wie bisher (11) nach dem hoch-empfindlichen Verfahren Braces mit einem variablen Azimutkompensator konstanter Retardation (drehbare $\lambda/30$ -Glimmerplatte) und einem drehbaren Halbschattenkeil nach Macé de Lepinay angestellt, während für die quantitative Bestimmung des *Ag-Dichroismus* Δk durch separate Ermittlung der beiden Absorptionskoeffizienten k_a , k_o zur Errechnung von $\Delta k = k_a - k_o$ die als zweckmässig gefundene Methode Berek's unter Verwendung seines Mikrophotometers (14) gedient hat.

ERGEBNISSE UND DISKUSSION

Wie seit Holtfreter vielfach gefunden, ist in den *Kulturen* das Auswachsen von glatten Muskelzellen zu beobachten. Zahlreich sind aber auch degenerierende Muskelzellen vorhanden, welche durch Rückbildung der Myofibrillen sich morphologisch Fibroblasten nähern. In Bestätigung zu Versuchen Magliozzis können mittels 5 % Citronensäure einzelne Muskelfasern isoliert werden. Der Zellkern ist in ausgestreckten Fasern länglich, kann aber in kontrahierten sphäroidale Form annehmen. *Phasenmikroskopisch* sind auch die feinen Myofibrillen gut zu erkennen, und selbst in plasmareicheren Zellen treten nicht nur die dickeren Grenzfibrillen am Rande hervor. Gelegentlich beobachtete knotige Verdickungen an den glatten Muskelzellen müssen wohl auf unregelmässige Kontraktion als Folge der mannigfachen technischen und Versuchseingriffe zurückgeführt werden.

Im *polarisierten Licht* zwischen gekreuzten Nicols zeigen die Explantate „la presenza di lunghe miofibrille fortemente birifrangenti“ (6). Die Anisotropie der untersuchten Myofibrillen erweist sich wie in andern Muskeln als zur Längsrichtung positiv einachsige (Abb. 1). Die Polypeptidketten liegen

also auch hier in der Faserrichtung. Aus der ermittelten Grössenordnung der $\Delta n = 2,0 \times 10^{-3}$ bis $2,9 \times 10^{-3}$ ($\pm 0,05 \times 10^{-3}$) darf vielleicht gefolgert werden, dass die Polypeptidketten ziemlich stark tordiert liegen. Eine Abschwächung der Δn , welche während der manchmal in der Kultur beobachteten Kontraktion erwartet wurde (16, 17), ist in keinem Falle mit Sicherheit konstatiert worden, so dass die Annahme Buchthals (2), eine solche Dimi-

Fig. 1. Die der Länge nach auslöschenden Myofibrillen bei Untersuchung glatter Muskeln zwischen gekreuzten Nicols.

Fig. 2. Die Zunahme der Δn der Myofibrillen nach Dehnen des Explantats.

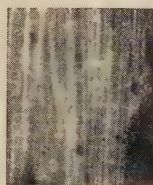


Fig. 1.

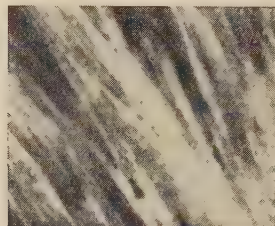


Fig. 2.

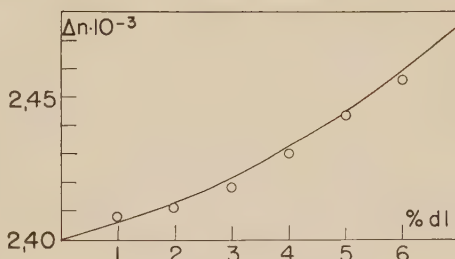


Fig. 3. Zunahme der Δn der Myofibrillen in Abhängigkeit vom Dehnungsgrade % dl.

nution der Anisotropie kontrahierender Muskelfasern sei nur auf die Wirkung von Säurebildung auf die Myoproteine zurückzuführen und gar nicht im Wesen mit der Kontraktion verknüpft, durch die Befunde gestützt wird.

Dagegen ist mit *experimenteller Dehnung* der Explantate und damit der in ihnen gelegenen Muskelfasern eine messbare Zunahme der Δn verbunden (Abb. 2), wenn auch zu sicherem Nachweise eine empfindliche Messungsmethode gehört. Einer Dehnung der Ausgangslänge um ca. 5 % entspricht dabei eine Anisotropiezunahme um 1,5 bis 2,2 % des Initialwertes, d. h. ein Betrag, welcher eben noch über der erreichbaren Streubreite der Messunsicherheit (s. oben) liegt (Abb. 3). Polarisationsoptisch und zumal nach Österbergs *polanret method* der Kombination von Phasen- und Polarisationsmikroskopie (s. oben) lässt sich aus beobachteten *Spannungstrajektorien* entnehmen, wie in dem System ausgepflanzter Kulturen in einen elastischen Spannungszustand versetzende Kräfte sich auf die Zellen der Explantate übertragen und ihre morphologische Gestaltung und die Migrationsprozesse

beeinflussen. Die Zugwirkung der Spannung erweist sich kausal-mechanisch als morphogenetischer und funktioneller Faktor und macht das Vermögen lokaler Um-Orientierung aller Texturgestaltungen und deren labile Adaptation an die „Erfordernisse“ der den genannten Wirkungen subordinierten glatten Muskelzellen verständlich (14 a).

Die positive Δn der Explantate wird durch *dichroitische Ag-Färbung* messbar herabgesetzt. Ein solcher Effekt kann nur als Kompensation durch negative Anisotropie der Ag-Leptonen verstanden werden, d.h. die gemessenen Besonderheiten im Absorptionsverhalten der Objekte sind wesentlich nicht auf diese und ihre Mischkörpurnatur, sondern auf die ein- oder aufgelagerten metallischen Leptonen zurückzuführen. Wie bei Absorptionen überhaupt nimmt auch der Δk glatter Muskelfasern mit der Grösse der vom Licht durchlaufenen Schicht zu. Wenn durch Gebrauch von Lifa-Filtern monochromatisches Licht verschiedener Wellenlängen zum Durchleuchten des Präparats benutzt wird, lässt sich ein Fortschreiten der Herabsetzung der positiven Δn von den längeren zu den kürzeren λ -Werten konstatieren (15). Bei *partieller Ag-Imprägnation* des Explantats, etwa durch teilweises Abdecken der Objekte in der erwähnten Art, treten auch bei den untersuchten glatten Muskeln an der Übergangszone anomale Farbabstufungen zwischen dem bei Gebrauch des Kompensators sofort erkennbaren stark (positiv) doppelbrechenden unbehandelten Teilen des Explantats und den infolge Kompensation der Ag-Leptonen entsprechend schwächer (positiven) anisotropen Teilen der dichroitisch gefärbten Partien des Präparats auf (15). Die Reduktion der positiven Δn führt aber nur selten soweit, dass es zur Inversion des optischen Charakters der Anisotropie kommt.

Wenn die ausgepflanzten Kulturen nach der Ag-Imprägnation einer *Dehnung* unterzogen werden, müssen die ein- oder aufgelagerten Ag-Leptonen an den anisodiametrischen Myofibrillen ziemlich gleichsinnig orientiert werden. Trotzdem aber lässt sich der Δk durch das Dehnen nicht wesentlich steigern. Hingegen kann man durch den Dehnungsversuch an Explantaten, welche gelegentlich trotz ausreichender Ag-Überschwemmung keinen deutlichen Δk zeigen, solchen in der gewohnten Intensität hervorrufen, d. h. die Parallelisierung der Leptonen der Myofibrillen glatter Muskelfasern ist dennoch mit einer Δk -Zunahme verbunden. Die Grössenordnung dieses Effekts lässt allerdings wahrscheinlich erscheinen, dass der Δk auch in diesen Fällen wesentlich von den Ag-Leptonen und deren Ausrichtung abhängt. Eine diffuse Verteilung der Ag-Teilchen in der glatten Muskulatur kann ich entgegen Voigt (20) nicht annehmen. Die Bedeutung des Ausrichtens der Leptonen für den Δk erkennt man auch daran, dass es ohne

Δn keinen Δk gibt und geben kann, so dass der besonders hochempfindliche Nachweis von Δk ein ausgezeichneter *Indikator für eine orientierte Leptonik* eines Substrats ist.

Ein Beweis für die Annahme, dass es sich bei dichroitischer Versilberung um einen *Adsorptionsvorgang* handelt, wird sich nicht leicht führen lassen, doch kann man experimentell zeigen, dass eine solche Deutung mit den

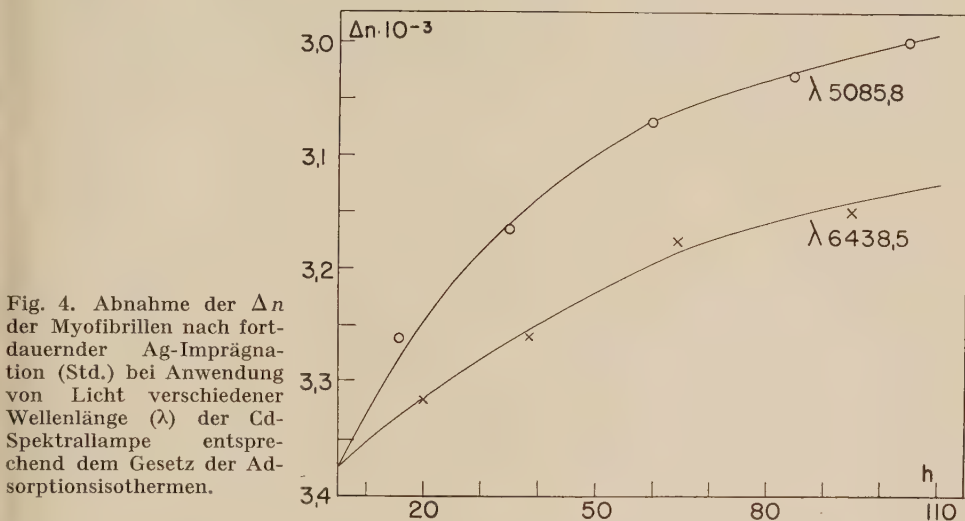


Fig. 4. Abnahme der Δn der Myofibrillen nach fort-dauernder Ag-Imprägnation (Std.) bei Anwendung von Licht verschiedener Wellenlänge (λ) der Cd-Spektrallampe entsprechend dem Gesetz der Adsorptionsisothermen.

Versuchsergebnissen keine Diskrepanz bildet. Wenn man etwa die Werte der Δn invers auf der Ordinate, die Zeit (Stunden) auf der Abszisse aufträgt, so lässt sich die Änderung der durch den Δk reduzierten Δn als Funktion der Zeit ermitteln (Abb. 4) und diese Kurve als in Übereinstimmung mit den Adsorptionsgesetzen nachweisen, d.h. die Adsorptionstheorie vermag an den Δn -Werten die Reduktion durch polarisationsoptisch negative Ag-Leptonen zu beschreiben. In welcher Grössenklasse die adsorbierenden Leptonen sind, ob sie Atome, Molekeln oder mit elektrischen Ladungen ausgezeichnete Molekelaggregate (Micellen) darstellen, muss allerdings unbeantwortet bleiben; daher mag der nichts darüber präjudizierende Terminus „Leptonen“ berechtigt sein.

Nach dem Ergebnis mannigfaltiger Versuchsanstellungen ist der Ag-Dichroismus wesentlich durch die metallischen Leptonen bedingt, deren adsorptive Bindung allerdings eine Ausrichtung erfordert, durch welche anscheinend eine polarisationsoptisch negative allotrope Modifikation der nach Grösse und Natur noch ungeklärt gebliebenen Ag-Leptonen hervorgerufen wird.

Ob es sich dabei trotzdem um die Bildung hexagonaler Ag-Teilchen handelt, wie Voigt (20) meint, oder ob räumlich bedingte Spannungen im molekularen Aufbau des Ag-imprägnierten Mischkörpersystems für die Produktion der geforderten allotropen Modifikation des Ag ausreichen (15), bleibe vorerst dahingestellt. Jedenfalls dürfte die Ag-Imprägnation trotz ihrer wesentlichen Abhängigkeit von den metallischen Leptonen *auch von Eigenschaften des Substrats*, insbesondere der physikalischen Natur seiner anscheinend besonders wirksamen (5) Fibrillen beeinflusst werden, und es dürfte eine direkte Relation zwischen Strukturdicke bzw. Lumen der interleptonischen Räume und der Intensität des Δk (resp. der Teilchengröße des Ag) bestehen (18, 21).

SUMMARY

When observed by phase microscope or between crossed nicols smooth muscles *in vitro* show distinct myofibrils and a *birefringence* up to $2,9 \times 10^{-3}$.

During contraction the birefringence of the muscles does not decrease, but after extension it increases moderately.

By embedding leptonic silver rodlets in muscle systems, the observed *dichroism*, indigo blue — straw yellow, produces a diminution of the birefringence, and in cases of partial metal impregnation there occurs anomalous gradation of colours.

Further the influence of extension on the dichroism is investigated as well as the possibility of interpreting the *silver impregnation* as an adsorption process.

Dichroism depends essentially on the silver leptones of a particular allotropic modification, but also on the orientation of the substratum, and the phenomenon is specially promoted by occurrence of fibrils.

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A STUDY OF THE HORMONAL RELATIONS OF EPIDERMAL MITOTIC ACTIVITY *IN VITRO*

I. TECHNIQUE

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THE study of hormone action *in vitro* is a comparatively new field of research. It is, however, full of promise, and reference to the work of Cori (9) on the relation of insulin and of growth hormone to carbohydrate metabolism will indicate the kind of advance which is possible. As regards the hormonal relations of mitotic activity, little or nothing appears to have been attempted unless the various studies on the growth *in vitro* of uterine and other similar tissues are considered. Such studies, using oestrogenic and pituitary hormones, have been made by numerous observers, and of the results obtained, the most important appear to be those of Gaillard (10). However, in all these cases the culture medium used was a highly complex mixture containing such fluids as embryo extract or serum. Clearly media of these kinds must contain large numbers of unknown factors, several of them hormonal, so that the results obtained may be difficult or even impossible to evaluate.

For this reason a number of attempts have been made to devise a saline medium in which mitotic activity may continue at least for several hours (7). As a first result, it has been possible to devise experiments which emphasise the critical importance of energy production to epidermal mitotic activity (8), and a review of this whole subject has recently been made (5). Epidermal mitotic activity only continues actively in saline culture media when some such substrate as glucose or lactate is provided, and when there is an oxygen gas phase.

From this conclusion it seemed possible, on the one hand, that a hormone which is known to affect the production of energy might also be found to affect mitosis and growth, while, on the other hand, a hormone which is known to affect mitosis and growth might also be found to affect carbohydrate metabolism. An investigation was therefore planned to determine the effects of a number of hormones on carbohydrate metabolism and on mitotic activity. In order to obtain the most precise information possible it

was planned to perform all these experiments *in vitro*, and until more information was obtained, to postpone the study of the more complex conditions *in vivo*.

However, before work could begin, it was found that a number of preliminary observations were necessary because, although unknown factors had been eliminated from the culture medium, they could not be so easily eliminated from the test tissue itself. The present paper is concerned with these preliminary observations, which constitute an analysis of certain aspects of the physiological condition of the host animals in relation to the behaviour *in vitro* of pieces of tissue taken from them. The aim has been to define as closely as possible what may be called a standard test animal from which standard tissue fragments may be obtained. Until this was done an accurate examination of hormonal action *in vitro* could not be attempted.

MATERIAL AND METHODS

The mice. As in previous work, all the mice chosen for study were albinos of the Kreyberg's WLL strain, and unless otherwise stated, only adult male mice of between 3 and 6 months of age were included in the experiments. Since weaning all the mice had been regularly given more food (a commercial rat cake with added cod liver oil) than they could eat. When adult males are fully fed, they are subject only to those diurnal variations in the mitotic rate which are associated with exercise and rest (2, 3). In order to maintain a regular diurnal rhythm, all the mice used were accustomed to a rigid routine of feeding and handling which ensured that they were disturbed only in the morning and that they were always asleep in the afternoon.

Juvenile mice, of less than 3 months old, were never used because their pattern of epidermal mitotic activity is known to differ from that of an adult (4), and because their peculiarities have not yet been investigated in detail. The use of old mice was also avoided for a similar reason.

The animals were killed without the use of an anaesthetic, and it was found that the most convenient methods were pitching or neck-pulling.

The ear epidermis. Immediately after the death of the mouse, the ears were detached and their thin peripheral regions were cut into small pieces, each about 2×4 mm, by means of a very sharp scalpel. It has already been calculated that with ear fragments of this size there is a relatively rapid and uniform penetration of nutrients and gases so that an equilibrium is established within the first hour of incubation.

Biochemical standards of cleanliness were maintained throughout, but no attempts were made to obtain aseptic conditions. It was found in practice that the 5 hours duration of each experiment was insufficient for any significant bacterial growth.

The culture technique. The ear fragments were incubated in groups of five (one from each of five mice) in a Warburg flask containing 4 ml of the phosphate-buffered saline medium devised by Bullough and Johnson (7). To enable the development of active mitosis in the Malpighian layer isotonic glucose was added to give

the optimum concentration of 0.02 M, and the gas phase was oxygen. The whole was maintained at 38°C and gently rocked. When they were first inserted the ear fragments usually showed a tendency to float on the surface of the medium, but as their natural covering of sebum melted they could be made to sink by active shaking.

To obtain an accurate estimate of the mitotic activity developing during the course of each experiment, colchicine was used. In each case, 0.016 mg colchicine dissolved in 0.04 ml normal saline was introduced into the side-arm of each flask. When, after 1 hr., all the mitoses originally present in the epidermis were judged to have passed through the metaphase, the colchicine was washed into the main vessel. The incubation was then continued for a further 4 hrs. during which time the colchicine arrested all newly-developing mitoses in the metaphase.

Histological and statistical techniques. The details of these techniques have already been described by Bullough and Johnson (7), and they need not be repeated here. It may be stated briefly that the ear fragments were fixed in Bouin's alcoholic fluid (also known as Duboscq-Brasil) embedded in ester wax, and stained in Ehrlich's haematoxylin and eosin. The numbers of mitoses, arrested in the metaphase, were counted in unit lengths of 1 cm of sections of epidermis cut 7 μ thick.

OBSERVATIONS

The effects of exercise and rest

From an analysis of the diurnal cycles of epidermal mitotic activity *in vivo*, Bullough (2, 3) concluded that muscular exercise inhibits epidermal mitotic activity, and that this may be due to the diversion of glucose to the muscles. Thus when a mouse is awake its epidermal mitotic rate falls to a low level, and when it is forced to take prolonged exercise in a slowly revolving box, epidermal mitosis is almost eliminated. It therefore appeared possible that the behaviour of an ear fragment *in vitro* might be influenced by whether the mouse was awake or asleep at the time of its death. It was in order to avoid this possible complication that the mice used by Bullough and Johnson (7, 8) were killed towards 14.00 hr., when they were all resting.

To test the importance of this point three groups each of 5 adult male mice were killed at 14.00 hr. The mice of the first group, the controls, were asleep at this time. The mice of the second group had been wakened by removal to a new box at 13.00 hr., and it should be noted that such a transfer always results in two or three hours of wakefulness while the animals investigate their new surroundings. The mice of the third group had been put into a slowly revolving box (one revolution in 4 minutes) at 09.00 hr., and so had been kept exercising for a period of 5 hr. Ear fragments from these animals were incubated in phosphate-buffered saline and the results are given in Table I.

TABLE I

The average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections 7 μ thick of ear epidermis cultured for 4 hr. at 38°C in phosphate-buffered saline and an oxygen gas phase. Each figure is the average of 5 observations.

Substrate	Condition of donor		
	asleep	awake	5 hr. exercise in revolving box
None	1.2 ± 0.24	1.0 ± 0.27	1.3 ± 0.40
0.02 M glucose . . .	6.9 ± 0.36	7.3 ± 0.35	7.7 ± 0.93

The condition of the epidermis at the time of its separation from the mouse was also checked, and it was found that the average number of mitoses in the standard unit of tissue in the sleeping mice was about 9, in the mice which were awake about 5, while in the exercising mice no mitoses at all were discovered. However, it is quite clear from Table I that these initial differences had no effect whatever on the results obtained, and it is therefore obvious that in experiments of this kind mice may be used with equal hope of success at any time of the day.

The effects of nutrition

In view of the evidence provided by Bullough and Eisa (6) that epidermal mitotic activity is powerfully depressed when the diet is inadequate, the influence of the animals own carbohydrate reserves on the potential epidermal mitotic activity of the separated ear fragments was next examined. Two experiments were performed, the first involving a reduction in the carbohydrate reserves, and the second involving an increase. In the first experiments, groups each of 5 adult male mice were deprived of all food, but not of water, for periods of 12 hr., 24 hr., and 36 hr. The mice were then killed at the same time as 5 normally fed controls, and their ear fragments were incubated in phosphate-buffered saline, both with and without the addition of glucose as substrate. This experiment was repeated, and the combined results are shown in Table II.

A check was also made on the condition of the epidermis at the time of its separation from the mouse, and in all the starved groups mitotic activity was much reduced. In the mice starved for 36 hr. no epidermal mitoses were found at all. This lack of mitotic activity, however, did not prevent a full return to normal when the ears were separated from the body. Indeed

TABLE II

The average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections 7 μ thick of ear epidermis cultured for 4 hr. at 38°C in phosphate-buffered saline and an oxygen gas phase. Each figure is the average of 10 observations.

Substrate	Condition of donor			
	normal	starved for		
		12 hr.	24 hr.	36 hr.
None	2.9 $\pm$ 0.43	4.0 $\pm$ 0.45	3.8 $\pm$ 0.26	4.2 $\pm$ 0.64
0.02 M glucose .	8.5 $\pm$ 0.78	10.1 $\pm$ 0.83	10.7 $\pm$ 0.54	11.1 $\pm$ 0.92

TABLE III

The average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections 7 μ thick of ear epidermis cultured for 4 hr. at 38°C in phosphate-buffered saline and an oxygen gas phase. Each figure is the average of 5 observations.

Substrate	Condition of donor			
	normal	previously injected with		
		10 mg glucose	20 mg glucose	40 mg glucose
None	1.8 $\pm$ 0.16	5.3 $\pm$ 0.50	11.2 $\pm$ 0.65	12.1 $\pm$ 0.58
0.02 M glucose .	6.8 $\pm$ 0.21	7.4 $\pm$ 0.32	10.8 $\pm$ 0.62	12.3 $\pm$ 1.18

the mitotic activity observed in the ear fragments taken from starved mice was in all cases above normal, but the reason for this is at present unknown.

A second experiment was then attempted in which the carbohydrate reserves of the mice were increased by subcutaneous injections of isotonic solutions of glucose. Again groups each of 5 adult male mice were used, the controls being injected with 0.5 ml isotonic saline. The mice in the three experimental groups received respectively 10 mg, 20 mg, and 40 mg of glucose, and for comparison with these figures it may be noted that Bissinger, Lesser, and Zipf (1) found that the carbohydrate content of a 20 g mouse after an 18 hr. fast is approximately 20 mg of glycogen and 20 mg of glucose. No figures for a normal mouse have been discovered, but from this evidence it would appear that the amounts of glucose injected were relatively massive.

The injections were given at 10.00 hr., and the killing of the mice began at 11.00 hr. so that the whole experiment was set up by 11.30 hr. The results are shown in Table III.

The mice were awake and active at the time they were killed, and no differences were observed between the relatively low rates of mitotic activity

then present in the various groups. During their subsequent incubation the ear fragments from the control mice behaved in the usual manner, slight mitotic activity developing when no substrate was provided and much mitotic activity developing when glucose was added. However in those mice previously injected with glucose the rate of mitosis developing *in vitro* was unusually high whether glucose was present or not.

It appears that at least two factors may have been involved in producing the result. In the first place it seems probable that the carbohydrate reserves of the ears were so greatly augmented by the previous glucose injections that the presence or absence of any further glucose in the culture medium ceased to have any importance. If this is true one conclusion which follows is that the carbohydrate reserves of the epidermis are not washed out even after several hours *in vitro*. If these reserves were in the form of glucose it might be expected that they would wash out, and their failure to do so may be taken as an indication that they are in the form of phosphorylated derivatives or of glycogen.

The second factor which may be involved is hormonal. Following the massive injections of glucose it can be expected that the mice must have reacted by a greatly increased secretion rate of insulin. The effect of insulin on epidermal mitosis is in the process of investigation, but since it is a hormone which is well known to influence carbohydrate metabolism, it may well be found to influence the rate of cell division too.

These various experiments involving starvation and glucose injection indicate that the most drastic treatment is necessary before the effects become apparent in any culture experiments. It is therefore probable that within normal limits the nutritional state of the animals used is unimportant.

The effects of shock

The possibility that significant quantities of a variety of hormones and of other active substances may be introduced into these experiments within the ear fragments themselves cannot be discounted, particularly in view of the observations of Cori (9) that isolated intact muscle retains the hormonal balance as it existed in the body at the time of its separation, the wash-out effects being negligible. In adult mice maintained for experimental purposes the two most obvious groups of hormones which might be suspected of exerting an influence in this way are those of the adrenal complex on the one hand and of the gonadal complex on the other. The first may be brought into play during such stress or shock as may result from overcrowding, bad

TABLE IV

The average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections 7 μ thick of ear epidermis cultured for 4 hr. at 38°C in a phosphate-buffered saline and an oxygen gas phase. Each figure is the average of 6 observations.

Substrate	Condition of donor		
	normal	shocked by tourniquet application	shocked by injection 10 mg ATP
None	2.4 $\pm$ 0.23	2.8 $\pm$ 0.19	3.0 $\pm$ 0.45
0.02 M glucose . . .	8.2 $\pm$ 0.50	7.2 $\pm$ 0.38	7.9 $\pm$ 0.44

housing, or bad handling, and in view of previous results (11, 5) they might be expected to induce a mitotic depression.

To study the effects of shock a series of experiments was performed in which the mice were injured either by the removal of tight rubber bands from their hind legs, or by the injection of ATP (11). Experimental groups each of 6 adult male mice were used, and the results are shown in Table IV. The rubber bands were applied to the mice at 10.00 hr. and removed at 12.00 hr., when shock rapidly developed. The injected mice were each given 10 mg ATP also at 12.00 hr., and again shock rapidly developed. At 13.30 hr. the animals, then in a state of collapse, were killed, and the experiment was set up by 14.00 hr.

Sample ear fragments taken from the mice when they were killed at 13.30 hr. showed that while the sleeping controls had a typically high rate of epidermal mitotic activity, most of the shocked mice contained no epidermal mitoses at all, while the remainder contained very few. However, this mitotic inhibition rapidly disappeared from the ear fragments maintained in the culture medium so that at the end of the experiment almost as many mitoses were counted in the two experimental groups as in the controls.

In view of this evident lack of effect, it was not considered necessary at this point to analyse the situation further. The precise modes of action of those hormones which may be expected to be secreted in excess during a state of stress or shock are to be considered in a subsequent paper.

The effects of the gonadal hormones

Considering next the effects of the gonadal hormones, it may be recalled that both oestrogens and androgens have been described as stimulating the mitotic rate of mouse epidermis (5). An absence or an abundance of these hormones in the test mice may therefore be reflected in the hormone content

TABLE V

The average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections 7 μ thick of ear epidermis cultured for 4 hr. at 38°C in a phosphate-buffered saline and an oxygen gas phase. Each figure is the average of either 5 or 10 observations.

Substrate	Condition of donor			
	5 mice in early dioestrus	5 mice in pro-oestrus	10 ovariectomised mice	10 ovariectomised mice injected 25 γ oestrone
None	0.8 $\pm$ 0.39	1.5 $\pm$ 0.16	0.9 $\pm$ 0.37	1.0 $\pm$ 0.27
0.02 M glucose .	4.6 $\pm$ 0.32	10.4 $\pm$ 0.58	3.1 $\pm$ 0.42	7.2 $\pm$ 0.49

of the ear fragments, and in particular, the state of the oestrous cycle may have a marked effect on results obtained *in vitro*. In the first experiments the effects of the state of the ovaries were determined in groups each of 5 adult mice. The first group of 5 mice was killed on the first day of dioestrus, when the epidermal mitotic rate is normally low; the second group of 5 mice was killed in pro-oestrus, when the epidermal mitotic rate is normally high; the third group consisted of 10 mice ovariectomised 7 days previously; while the fourth group contained 10 similarly ovariectomised mice each injected with 25 γ oestrone 12 hr. before killing. The experiments were set up as before, and the results are shown in Table V.

All the mice were killed when they were awake and active, and therefore all had a relatively low rate of epidermal cell division. However, the subsequent development of mitosis *in vitro* was evidently strongly influenced by the condition of the ovaries and by the injections of oestrone. Epidermis from mice with quiescent ovaries on the first day of dioestrus, like that from ovariectomised mice, developed a mitotic rate which was low compared with that shown by epidermis taken from mice with active ovaries in pro-oestrus or from mice which had been injected with oestrone.

This result is consistent with the theory that the natural oestrogens were carried into the experiment in the epidermis of the mice in pro-oestrus, that they did not wash-out during a period of several hours, and that they continued to stimulate epidermal mitosis in the way they normally do *in vivo*.

Conditions in the male mouse were next examined. Four-month-old animals were castrated, and allowed to recover for four days. The behaviour of the epidermis of three experimental groups was then compared. The first group consisted of 5 normal males, the second of 5 castrated males, and the third of 5 castrated males which during the preceding 24 hr. received two injections each of 0.25 mg testosterone propionate. The experi-

TABLE VI

The average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections 7 μ thick of ear epidermis cultured for 4 hr. at 38°C in phosphate-buffered saline and an oxygen gas phase. Each figure is the average of 5 observations.

Substrate	Condition of donor		
	Normal	Castrated	Castrated and injected twice with 250 γ testosterone propionate
None	0.9 $\pm$ 0.16	0.5 $\pm$ 0.12	1.0 $\pm$ 0.15
0.02 M glucose . . .	7.0 $\pm$ 0.84	5.8 $\pm$ 0.63	6.4 $\pm$ 0.72

ment was set up as before, and the number of mitoses developing in the incubated epidermis are shown in Table VI.

The results show a somewhat reduced mitotic rate in the castrated animals, but when the standard errors are taken into consideration it will be seen that the differences observed are not great.

GENERAL CONCLUSIONS

Having already devised a culture medium containing no unknown substances and capable of supporting active mitosis in fragments of mouse ear epidermis, a study has now been made of the more obvious factors, nutritional and hormonal, which may be introduced into the experiments inside the ear fragments themselves. As thorough an understanding of these factors as possible is a necessary preliminary to any *in vitro* analysis of the physiology of cell division. The main conclusions reached are as follows.

1. Although a pronounced diurnal cycle of mitotic activity exists in the mouse, the effects of this do not persist when separated epidermis is maintained *in vitro*. It follows that similar results can be obtained from ear fragments taken from mice at any hour of the day or night.

2. A consideration of the state of nutrition of experimental mice is also essential to an understanding of the epidermal mitotic activity *in vivo*, but within wide limits starvation or overfeeding is without effect on the subsequent mitotic activity of the epithelium *in vitro*. Conditions outside these limits are most unlikely to occur in any normal mouse colony.

3. Overcrowding, rough handling, or any other condition inducing stress has a strong action in depressing epidermal mitotic activity *in vivo*, but even shock so severe as to cause the complete collapse of the mice does not affect the rate of epidermal mitosis subsequently developing *in vitro*.

4. By contrast the effects of the female sex hormones may be considerable.

If the mouse is in early dioestrus with a minimum rate of oestrogen secretion, a low rate of cell division is also obtained in experiments *in vitro*, and conversely if the animal is in pro-oestrus with a high rate of oestrogen secretion, a high rate of cell division develops *in vitro*. Evidently the oestrogenic hormones do not wash out of the ear fragments, and so, remaining undiluted, they continue to exert their normal mitogenic action.

5. The androgenic hormones of the male mouse evidently have a far weaker effect. The evidence indicates, but does not prove, that the presence of these hormones in the ear fragments also exerts some slight stimulating effect on epidermal mitosis *in vitro*.

These results permit a definition of the most suitable type of mouse for use as a donor of ear epidermis. Since the presence or absence of sex hormones may exert a considerable effect on the results obtained, the ideal animal is a castrate. The epidermis taken from such a mouse, whether male or female, has been found to give results which are consistently uniform. However, in practice it has been found that the normal adult male, which has a relatively uniform rate of androgenic hormone secretion, can be used with almost equal success. The effect of the presence of androgenic hormones is only slight, and except in experiments involving the use of such hormones, it may be disregarded.

One warning must be given. The epidermal mitotic rate is violently increased by irritation or wounding, and only perfectly healthy ears can be used. If the ear fragments from an individual mouse give mitotic counts which are more than double the average for the rest of the group, the sections should be carefully examined for other signs of epidermal damage. In the later stages of healing such signs are not always easy to distinguish.

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A STUDY OF THE HORMONAL RELATIONS OF EPIDERMAL MITOTIC ACTIVITY *IN VITRO*

II. INSULIN AND PITUITARY GROWTH HORMONE

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It is well known from the work of Cori (9) and others that a number of hormones interfere at some point or other in the course of carbohydrate metabolism. The most important stimulators are generally recognised to be insulin, which facilitates the irreversible glucokinase reaction, and adrenalin, which hastens the phosphorylation of glycogen, while Cori also maintains that important inhibitors are the pituitary growth hormone and the adrenal glucocorticoid hormone, which both act, perhaps synergistically, on the glucokinase reaction. There are reviews on this subject by Krah1 (14), Verzá1 (20), and Young (21), and Verzá1 quotes evidence which throws doubt on Cori's conclusions regarding the action of the glucocorticoid hormone on glucokinase. This point will be considered in a future paper, and for the present attention will be confined to the question of insulin and of the growth or diabetogenic hormone of the anterior pituitary gland, the action of both of which on the glucokinase reaction is not in doubt.

Evidence has already been obtained to indicate the close dependence of epidermal mitotic activity on active energy production through the tricarboxylic acid cycle (7), and the conclusion has been drawn that epidermis is normally unable to absorb more than a small fraction of that glucose which is present in the intercellular fluid (4). It is therefore of interest to discover whether these two hormones can influence the rate of glucose absorption of the epidermal cells as they are known to do in the case of rat diaphragm muscle (14), and as they may also do in liver (8) and in adipose tissue (14). If they are active in this way they may also be expected to influence epidermal mitotic activity, and the present paper is therefore concerned with an attempt to throw light on the role played by insulin and growth hormone in the growth processes of the adult mouse.

MATERIAL AND METHODS

Details of the techniques employed have been described in a previous paper (5), but it may be re-emphasised that only adult male mice were used.

It remains only to add that the insulin was a specially purified sample kindly provided by Professor H. A. Krebs, while the growth hormone was prepared according to Wilhelmi's method by Organon Laboratories. Both were simply dissolved in the saline medium.

OBSERVATIONS

The effects of insulin

Insulin is well known to stimulate the rate of glucose uptake in rat diaphragm muscle and to increase the energy output (14). If it has a comparable action on mouse epidermis the result may be a stimulation of the mitotic rate. Two experiments were prepared to test the possible mitogenic power of this substance, and the combined results are shown in Table I.

TABLE I

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with 0.02 M glucose and an oxygen gas phase. Each figure is the average of 10 observations derived from two separate experiments.

Insulin concentration per 4 ml culture medium				
0	0.5 γ	5 γ	50 γ	250 γ
8.4 $\pm$ 0.41	11.1 $\pm$ 1.01	13.1 $\pm$ 0.68	14.5 $\pm$ 0.78	14.7 $\pm$ 1.07

Evidently insulin can act as a mitotic stimulant to mouse epidermis *in vitro*, and in the conditions of the present experiments the lowest fully effective concentration lies somewhere between 5 γ and 50 γ per 4 ml saline medium.

The question of the precise point or points of stimulus now arises, and working on the presumption that the insulin is acting through its well-known influence on carbohydrate metabolism, it may first be asked whether its action is on glycolysis or respiration. If the insulin stimulates a critical rate-limiting reaction, which normally hinders the efficient usage of glucose, this may lie either before or after the pyruvate stage. If it is before this stage it should prove possible to by-pass it by the use of L-lactate as substrate in place of glucose. Two experiments were carried out in this connexion. In the first the optimum concentration of L-lactate was determined, and the combined results of three experiments, using a total of 15 adult male mice, are shown in Table II.

From this it may be concluded first that the optimum lactate concentration

TABLE II

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7μ thick of ear epidermis incubated at 38°C in saline medium with various concentrations of substrate and an oxygen gas phase. Each figure is the average of 15 observations.

Substrate					
none	0.02 M glucose	0.01 M L-lactate	0.02 M L-lactate	0.03 M L-lactate	0.04 M L-lactate
1.4 ± 0.05	9.1 ± 0.76	12.9 ± 0.80	16.4 ± 0.98	9.0 ± 0.39	3.8 ± 0.40

TABLE III

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with an oxygen gas phase. Each figure is the average of 10 observations.

Substrate				
none	0.02 M glucose		0.02 M L-lactate	
	alone	+ 5 γ insulin	alone	+ 5 γ insulin
0.4 ± 0.06	6.5 ± 0.40	12.2 ± 0.83	13.8 ± 0.93	12.8 ± 1.12

approximates to 0.02 M, and second that the mitotic rate induced by optimum lactate is almost double that induced by optimum glucose. This second conclusion is exactly what would be expected if some pre-pyruvate reaction is normally rate-limiting in the process of epidermal energy production.

The second experiment involved a comparison of the action of insulin with glucose and L-lactate as substrates, the results being given in Table III.

These results confirm the supposition that a rate-limiting reaction, which may be stimulated by insulin, exists somewhere between extra-cellular glucose and intra-cellular pyruvate, and in view of the known action of insulin the question immediately arises whether this may in fact be the hexokinase reaction.

Attempts were next made to by-pass the hexokinase reaction with the use of phosphorylated intermediates as substrates in place of glucose. The first attempt involved the use of glucose-1-phosphate, the sample used being an unpurified potassium salt, and the results of the experiments involving 10 adult male mice are given in Table IV.

High mitotic activity was obtained with glucose, but with glucose-1-phosphate activity was low. With the highest concentration there were some signs of damage to the epidermal cells. It is doubtful from these figures

TABLE IV

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in saline medium with glucose or glucose-1-phosphate and an oxygen gas phase. Each figure is the average of 10 observations.

Substrate				
none	glucose	glucose-1-phosphate		
	0.02 M	0.01 M	0.02 M	0.03 M
2.8 $\pm$ 0.31	13.8 $\pm$ 0.85	3.6 $\pm$ 0.56	3.4 $\pm$ 0.55	0.7 $\pm$ 0.16

TABLE V

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in saline medium with glucose, glucose-1-phosphate, or fructose-1,6-diphosphate and an oxygen gas phase. Each figure is the average of 10 observations.

Substrate							
none	glucose	glucose-1-phosphate			fructose-1,6-diphosphate		
	0.02 M	0.01 M	0.02 M	0.03 M	0.01 M	0.02 M	0.03 M
2.5 $\pm$ 0.37	9.5 $\pm$ 0.80	2.2 $\pm$ 0.31	2.2 $\pm$ 0.40	0.9 $\pm$ 0.25	1.2 $\pm$ 0.08	0.5 $\pm$ 0.06	0.4 $\pm$ 0.07

whether any glucose-1-phosphate penetrated the cells since the mitotic activity obtained with the two lower concentrations may have been entirely due to energy reserves within the cells themselves. However, since this sample had not been carefully purified, it seemed possible that the low activity obtained may in fact have been due to some contamination, for instance to slight traces of barium.

The experiment was therefore repeated with a purer sample of the potassium salt of glucose-1-phosphate, which was provided by Professor H. A. Krebs. Ten adult male mice were again used and the results are given in Table V. At the same time an opportunity arose to test the action of the magnesium salt of fructose-1,6-diphosphate, and these results are also included in the table.

The results with glucose-1-phosphate are similar to those in Table IV. Evidently this substance did not penetrate the cells to provide a source of energy, and with the highest concentration an inhibition developed. The limited mitotic activity observed was evidently based on energy supplies within the epidermal cells, as in the control group provided with no substrate.

TABLE VI

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in saline medium with glucose or fructose and an oxygen gas phase. Each figure is the average of 10 observations.

Substrate				
none	glucose 0.02 M	fructose		
		0.01 M	0.02 M	0.03 M
2.8 $\pm$ 0.31	10.8 $\pm$ 0.85	10.5 $\pm$ 0.43	15.0 $\pm$ 0.84	8.0 $\pm$ 0.86

The fructose-1,6-diphosphate gave even poorer results, and it is clear that at all concentrations its effect was to inhibit mitosis. This need not necessarily be taken to indicate that the substance penetrated into the cells for the inhibition may have been due to some surface reaction.

At this point the attempt to supply phosphorylated intermediates as substrates was abandoned, and a new attempt to by-pass the hexokinase reaction was made with the use of fructose. It is known that liver and muscle contain separate fructokinase and glucokinase systems (9), and it was considered that the same might possibly be true of epidermis, although in brain a single generalised hexokinase can evidently catalyse the phosphorylation of both fructose and glucose (19).

The activity of fructose as a substrate for epidermal mitosis was first studied with ear fragments taken from 10 adult male mice, and the results are shown in Table VI.

The result of this experiment was to show clearly that fructose is at least as efficient, and possibly considerably more efficient, as a substrate for epidermal energy production than is glucose. From a comparison with previously published work (6) it can also be stated that both sugars have an optimum concentration of about 0.02 M. It follows either that the phosphorylation of the two sugars is accomplished by a single hexokinase or that the epidermis contains both a glucokinase and an equally efficient, or even more efficient, fructokinase.

If only one generalised hexokinase exists in the epidermal cells insulin should facilitate the phosphorylation and utilisation of glucose and fructose in equal degree. If there are separate gluco- and fructokinases it may only influence the former (9). To test these alternatives the experiments recorded in Table VII were performed.

These results confirm the impression that fructose is a considerably more efficient substrate than glucose for the support of epidermal mitosis *in vitro*.

TABLE VII

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in saline medium with an oxygen gas phase. Each figure is the average of 5 observations.

Substrate 0.02 M glucose				
alone	0.5 γ insulin	5 γ insulin	50 γ insulin	250 γ insulin
9.1 $\pm$ 0.52	14.3 $\pm$ 1.19	17.7 $\pm$ 0.96	18.7 $\pm$ 0.88	17.0 $\pm$ 0.89
Substrate 0.02 M fructose				
alone	0.5 γ insulin	5 γ insulin	50 γ insulin	250 γ insulin
15.2 $\pm$ 0.87	15.0 $\pm$ 0.73	14.6 $\pm$ 0.71	15.2 $\pm$ 1.06	14.7 $\pm$ 0.99

However the two most important conclusions emerge from the fact that, while insulin can stimulate the rate of mitosis when the substrate is glucose, it does not do so when the substrate is fructose. From this it would appear first that the hexokinase which induces the phosphorylation of glucose is not the same as that which induces the phosphorylation of fructose. There appear, therefore, to be separate gluco- and fructokinases in mouse epidermis, an observation which is unexpected in view of the fact that in normal circumstances little or no fructose can reach the epidermal cells.

The second conclusion which follows is that, in stimulating mitotic activity in the presence of a substrate of glucose, insulin probably acts, as it does in other tissues, by facilitating the reaction glucose $\rightarrow$ glucose-6-phosphate. It is, of course, theoretically possible that the isomerase reaction glucose-6-phosphate $\rightarrow$ fructose-6-phosphate is the critical one, but in view of results obtained with rat diaphragm (14), it is felt that this is improbable.

The results obtained so far are in agreement with the theory that the normal epidermal cell has a low rate of division because it lacks the ability to produce significant amount of energy. This in turn is due to its inability to absorb sufficient glucose because of the slowness of the rate-limiting reaction glucose $\rightarrow$ glucose-6-phosphate. The mitogenic action of insulin may thus be wholly due to its influence over the one enzyme system.

The effects of growth hormone

Cori (9) has described the inhibiting effects of pituitary growth hormone on the glucokinase reaction, and Krahl (14) in his review has stressed the

TABLE VIII

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections $7\ \mu$ thick of ear epidermis incubated at 38°C in 4 ml saline medium with various substrates and with an oxygen gas phase. Each figure is the average of 5 observations. The number of mitoses observed in the absence of any substrate was 1.7 ± 0.35 .

Substrate 0.02 M glucose				
alone	growth hormone			
	10 γ	100 γ	500 γ	1000 γ
8.4 ± 0.86	7.8 ± 0.65	5.4 ± 0.39	3.0 ± 0.27	2.5 ± 0.24
Substrate 0.02 M fructose				
alone	growth hormone			
	10 γ	100 γ	500 γ	1000 γ
11.8 ± 0.24	13.1 ± 1.73	10.9 ± 0.98	11.4 ± 1.12	11.7 ± 0.90
Substrate 0.02 M L-lactate				
alone	growth hormone			
	10 γ	100 γ	500 γ	1000 γ
11.4 ± 0.45	12.9 ± 0.81	11.4 ± 0.62	12.6 ± 0.39	12.0 ± 0.45

normal antagonism which exists between this hormone and insulin. In the previous experiments on which these conclusions are based, the growth hormone was injected intraperitoneally many hours before the rats were killed for the removal and incubation of their diaphragms. In the present experiments the growth hormone was added instead to the culture medium.

Following a preliminary experiment which demonstrated the paradoxical fact that growth hormone preparations do in fact inhibit epidermal mitosis, the comprehensive experiment recorded in Table VIII was performed.

These figures confirm the surprising conclusion that, with glucose as substrate, growth hormone is an epidermal mitotic inhibitor, at least in the conditions of this experiment. However, no signs of any inhibition are evident when either fructose or L-lactate is used as substrate. This is in agreement with the theory that growth hormone inhibits selectively the glucokinase reaction, and that this, through a limitation of the rate of epidermal energy production, also inhibits the rate of epidermal mitosis.

With insulin stimulating epidermal mitosis through its facilitation of the

TABLE IX

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with 0.02 M glucose and an oxygen gas phase. Each figure is the average of 5 observations. The numbers of mitoses observed in the absence of the substrate was 0.3 ± 0.12 .

Without hormone	50 γ insulin	500 γ growth hormone				
		alone	0.5 γ insulin	5 γ insulin	50 γ insulin	500 γ insulin
6.7 ± 0.48	9.2 ± 0.73	1.0 ± 0.08	0.9 ± 0.21	2.1 ± 0.32	2.8 ± 0.15	3.5 ± 0.30

TABLE X

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with 0.02 M glucose and an oxygen gas phase. Each figure is the average of 5 observations. The number of mitoses observed in the absence of the substrate was 1.1 ± 0.18 .

Without hormone	500 γ growth hormone	50 γ insulin				
		alone	10 γ growth hormone	100 γ growth hormone	500 γ growth hormone	1000 γ growth hormone
8.1 ± 0.29	2.9 ± 0.36	11.9 ± 0.54	10.8 ± 0.50	7.3 ± 0.22	4.5 ± 0.25	4.4 ± 0.28

glucokinase reaction, and with growth hormone depressing epidermal mitosis through its inhibition of the same reaction, it becomes of interest to discover how far each of these two hormones can counter the action of the other. The two experiments recorded in Tables IX and X were designed to this end.

The results show clearly that the severe mitotic depression induced by 500 γ growth hormone can be to some extent offset by large doses of insulin, whilst conversely the stimulus of 50 γ insulin can be eliminated by large doses of growth hormone.

DISCUSSION

As mentioned above, evidence has already been reviewed to indicate that the epidermal cells of the mouse are normally able to absorb only a fraction of the glucose present in the intercellular space, whether in normal conditions or in the conditions of a tissue culture experiment (4). The results here recorded lend support to this view, and add the further information that this

may be partially or entirely due to the normal sluggishness of the glucokinase reaction. It appears that in the epidermis this reaction is, so to speak, poised midway so that it may be either stimulated by insulin or inhibited by growth hormone.

It is of interest to speculate on the actual location in the cell of the phosphorylating mechanism. On the one hand it is possible that glucose might diffuse passively through the cell wall to be phosphorylated by glucokinase situated perhaps on the mitochondria. On the other hand it is possible that the phosphorylating mechanism is situated in the cell wall so that glucose can gain entry to the cell only after it has been phosphorylated. There is no critical evidence available on this point, but such indications as there are seem to favour the second alternative.

One such indication is given by the fact that phosphorylated glucose derivatives are evidently unable to penetrate the cell wall. If they could do so there is no obvious reason why they should not enter into the normal metabolic pathway. If, however, glucose must normally be phosphorylated as it enters the cell, then it could easily be supposed that previous phosphorylation could prevent the accomplishment of the normal cell-surface reaction. A second argument comes from the observation that in tissue homogenates, the glucokinase system is maximally active (9). Evidently the destruction of the cell wall and the disorientation of the normal cell constituents upsets the delicate chemical balance as it normally exists. Other evidence in favour of the importance of the cell wall is offered by Goldstein, Huddleston, and Levine (11).

It is therefore possible that the stimulus of insulin and the inhibition of growth hormone are both cell surface reactions.

The fact that growth hormone has proved to be a mitotic inhibitor is so curious as to demand further consideration. The diabetogenic influence of the pituitary was first shown by Houssay and Biasotti (12, 13), while the diabetogenic action of purified growth hormone was first described by Cotes, Reid and Young (10). From this has come our present knowledge of the power of growth hormone to inhibit glucokinase. Different samples of growth hormone are equivalent in their power to stimulate the growth of intact rats and to inhibit glucose uptake in rat diaphragm *in vitro* (14, 21). However, highly purified growth hormone preparations, as tested by glucose uptake inhibition in excised diaphragm, are apparently inactive for several hours after they have been injected into rats, and this has led Krah1 to support the suggestion that a hormone "which is fully active for growth but inactive towards glucose uptake may be transformed in the body to an

active inhibitor of glucose uptake". This theory could be held to account from the relatively high concentrations of growth hormone which were required to depress epidermal mitosis in the present experiments because the concentration of the rapidly acting inhibitor, which is evidently high in crude pituitary extracts, may be greatly reduced in highly purified growth promoting extracts.

There may therefore exist a pituitary glucokinase inhibitor which is chemically closely related to growth hormone but which is distinct from it. This view is apparently supported by the results of Raben and Westermeyer (17), but attempts, such as those of Reid (18), to separate the two supposed hormones have so far failed. A full review of this subject has been published by Young (21), who concludes that there is no firm evidence at the moment to support this theory of the existence of two separate hormones.

It may alternatively be suggested that the administration of growth hormone to a whole animal is normally countered by the unusually active production of insulin, which is itself the real growth hormone. As long ago as 1933 Anselmino, Herold, and Hoffmann (1) demonstrated an increased islet area in sections of pancreas taken from rats treated with crude pituitary extracts, and more recently this observation has been confirmed (21). There is also evidence that injections of growth hormone raise the insulin content of the pancreas in rats (15, 16), which do not then develop diabetes, but not in dogs (3), which do become diabetic. There may therefore be grounds for supposing that growth hormone promotes growth only if the islets are capable of secreting sufficient insulin, and that otherwise it causes diabetes. Young (21) reviews evidence in favour of this, and of the further suggestion that the ability of the islets to respond by increased secretion is a faculty which is depressed or lost in the adult. If this is so it should be possible to promote the growth of a young hypophysectomised rat by insulin alone, and this has actually been done (2).

While these conclusions are not yet fully established, they are clearly in agreement with the present observations that insulin is an epidermal mitotic stimulant, while growth hormone, or possibly its diabetogenic contaminant, is antimitotic. Since the epidermal mitotic rate is primarily determined by the supply of intra-cellular glucose, which is in turn apparently a function of the degree of activity of the glucokinase reaction, the present results may be fully explained in terms of the known actions of these two hormones on this particular enzyme system. In the whole animal, however, the actions of these hormones in stimulating or inhibiting growth, in preventing or causing diabetes, must be more complex.

SUMMARY

1. In view of the known dependence of the epidermal mitotic activity of the adult male mouse on the rate of entry of glucose into the cells and of its subsequent conversion to energy, a study has been commenced of the *in vitro* effects on epidermal mitosis of hormones known to influence carbohydrate metabolism. The present paper deals with the action of insulin and of the pituitary growth hormone.

2. It has been demonstrated that, with glucose as substrate, insulin increases the number of mitoses which develop in a period of 4 hr. in mouse ear epidermis, while growth hormone acts as an inhibitor. When both hormones are introduced together they counteract each other. A detailed analysis of these phenomena has indicated that the point of action of both hormones is in the glucokinase system.

3. This evidence gives further support for the view that the one factor normally limiting the epidermal mitotic rate is the slowness of the glucokinase reaction. This reaction is normally poised mid-way, and is capable of being either stimulated or depressed.

4. These results are discussed particularly in relation to the possible manner whereby the so-called growth hormone, which may actually prove to be a growth inhibitor, nevertheless normally brings about an increase in body size.

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OBSERVATIONS ON THE NUCLEAR MEMBRANE WITH
THE ELECTRON MICROSCOPE¹

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A COMPLEX physical structure for the nuclear membrane is suggested by several recent studies with the electron microscope. As seen in thin sections the nuclear membrane of rat nerve cells consists of two electron dense layers separated by a less dense space, with a general tangential orientation of submicroscopic fibrils (5). Surface views of the nuclear membrane of *Amoeba* (2, 4) and the amphibians *Triturus* and *Xenopus* (3) show two layers, one apparently continuous and the other porous. The pores of *Amoeba* are about 1200 Å diameter and are in the inside layer of the membrane, while those of *Triturus* and *Xenopus* are about 500 Å diameter in the outside or cytoplasmic layer. The present study provides new evidence on the structure of the nuclear membrane of the salamander, *Triturus*.

The large nucleus (germinal vesicle) from oocytes of the salamander, *Triturus viridescens*, reaches a diameter of nearly 0.5 mm and can be isolated readily with jeweler's forceps. The nuclear membrane is a distinct skin which can be peeled off with little difficulty. For electron microscope observations the membrane has been mounted on conventional 200 mesh wire screens in a manner similar to that used by Callan and Tomlin (3). A nucleus, isolated in 0.1 M NaCl, is pipetted into a drop of the same medium on a screen. The liquid is drawn off carefully with a piece of filter paper until the nucleus is considerably flattened by the surface tension force. A drop of 2 per cent OsO₄ is next allowed to fall onto the preparation from a height of several millimeters. The nucleus is broken open by this procedure and the contents washed out. However, a piece of nuclear membrane remains attached to the screen, covering a dozen or more open grid spaces. The cytoplasmic side of the membrane is obviously toward the screen. After a fixation period of 10 minutes or more the OsO₄ is replaced by successive changes of distilled water. The wet screen is finally picked up and placed face down on a thin film of "Formvar" plastic floating in a dish of water. The grid and

¹ Aided by research funds from the Graduate School of the University of Minnesota.

plastic together are picked up by a downward sweep of a glass microscope slide. Some specimens are air dried at this point while others are dehydrated in a series of alcohols and dried by the CO_2 critical point method of Anderson (1). The latter method preserves the three dimensional architecture of the surface to best advantage, but the general similarity of air dried and CO_2 dried material indicates that air drying does not grossly distort the nuclear

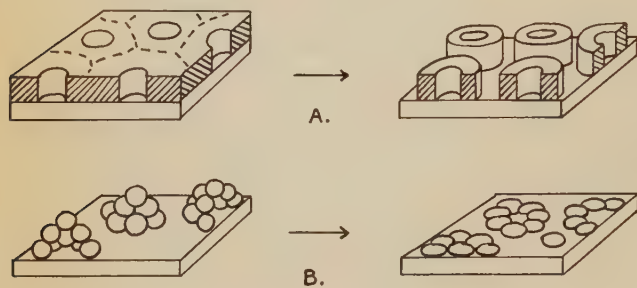


Fig. 1 a) Annuli produced by breakdown of a continuous porous sheet (3). b) Annuli, sometimes plugged in the center, produced by collapse of piles of small spheres.

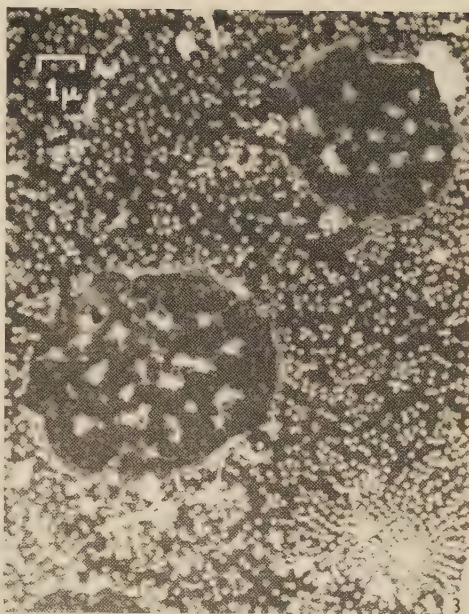
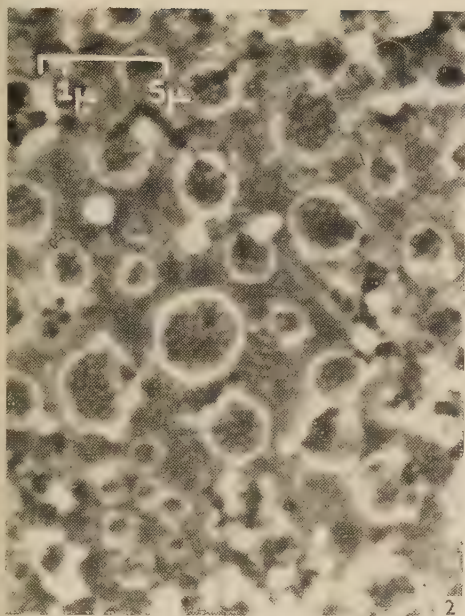
membrane. Specimens have been studied unshadowed or after shadowing with chromium at an angle of 30° by the method of Williams and Wyckoff (6).

The detail visible in an undried nuclear membrane using the light microscope alone is shown in Fig. 2 (bright phase contrast). The surface is covered with a large number of circular areas varying in diameter from one to several micra. Each circular area has a speckling of distinctly resolvable granules. Fig. 3, a low power electron micrograph, shows an exactly comparable structure. The granules in the circular areas are here seen to be clusters of still smaller granules. These smaller granules are also scattered outside the circular areas, but their regular distribution in these regions prevents visualization in the light microscope. The individual granules are about $1200 \text{ \AA}$ in diameter (0.12μ). Still higher magnification (Fig. 4) brings out the structure of the granules themselves. Many appear to be rather regular annuli, composed of eight to ten smaller masses arranged in a circle, though in other cases the center of the annulus may be plugged (arrow). The individual masses making up an annulus are roughly $400 \text{ \AA}$ in diameter, while the center of the annulus itself has a diameter of about $500 \text{ \AA}$. Some

Fig. 2. Surface view of nuclear membrane showing distinct granulation within circular areas of various sizes. Phase contrast microscope. 2 per cent OsO_4 , undried. $\times 3,420$.

Fig. 3. A similar region seen in the electron microscope. Granules in the circular areas are formed of clusters of still smaller granules, which are scattered individually over the rest of the membrane. 2 per cent OsO_4 , dried by Anderson's CO_2 method. Chromium shadow. $\times 6,950$.

Fig. 4. Individual granules at higher magnification are seen to be annuli, composed of eight to ten smaller masses. 2 per cent OsO_4 , air dried. Chromium shadow. $\times 88,000$.



membranes prepared by the CO₂ drying method suggest that the annuli are in part artefacts produced by air drying. After CO₂ drying the annuli are replaced by more or less hemispherical piles of spheres. If such piles were to collapse during the air drying process, they might well form the annuli seen; an annulus would be plugged if a sphere on top of the pile were pulled straight down. Metal shadowing shows that the annuli, or piles of spheres, are on the outside or cytoplasmic surface of the nuclear membrane.

The present observations confirm the results of Callan and Tomlin (3) on the nuclear membrane of *Triturus*, but suggest a different interpretation of the annuli. Callan and Tomlin considered the annuli to be breakdown products of a uniformly porous layer, as shown in Fig. 1a. The present interpretation of the annuli is shown diagrammatically in Fig. 1b. The reasons for preferring the latter interpretation are as follows:

1. The annuli are exceptionally uniform in size. If they form from the breakdown of a continuous porous sheet, the lines of weakness in that sheet must be quite regular.

2. There is no indication that adjacent annuli fit together like parts of a puzzle, as was suggested by Callan and Tomlin.

3. The clustering of annuli into groups visible with the light microscope is found in undried membranes (circular areas of Fig. 2). This observation suggests that the annuli already exist before preparation of the membrane for electron microscopic examination.

In summary, the nuclear membrane of *Triturus* oocytes consists of a continuous sheet upon which are scattered raised annuli. The annuli, on the cytoplasmic side of the continuous sheet, are approximately 1200 Å in diameter with a center 500 Å across. Each annulus is composed of eight to ten roughly spherical masses 400 Å in diameter. The annuli are scattered singly over the membrane except in circular areas where they occur in clusters. The circular areas and clusters are clearly visible in the light microscope.

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SPECTROPHOTOMETRIC STUDIES ON THE INTERACTION OF NUCLEIC ACIDS WITH AMINOACRIDINES AND OTHER BASIC DYES¹

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THE unique property of aminoacridine dyes to stain the nuclei of living cells (2, 3) has led to a consideration of the possible chemical interactions involved. Previous work has shown that this affinity for nuclei *in vivo* is based on the presence of desoxyribose- and ribosenucleic acids. The logical explanation for the affinity of aminoacridines for nucleoproteins is the formation of a chemical bond between the basic dye molecule and the nucleic acid components. A consequence of this assumption requires that either the aminoacridines are capable of displacing the protein component of the nucleoproteins or that there are free phosphoric acid groups present. But the problem still remains as to why other basic dyes do not have the ability to stain nuclei *in vivo*.

Recognizing that the protein components of the nucleus may have a profound effect on the affinity of basic dyes for nuclear material, the present study has nevertheless been limited to the role of the nucleic acids. The near ultraviolet and visible absorption spectra of mixtures of basic dyes, including many of the aminoacridines, with nucleic acids were measured. The changes in the absorption spectra were also determined for mixtures of representative dyes and nucleic acids under conditions of changing nucleic acid concentration and of changing pH.

It was shown by Kurnick (4) that the staining of the desoxyribosenucleic acid of the cell nucleus by methyl green is dependent upon the degree of polymerization of the acid. To test the applicability of this hypothesis to the staining by aminoacridines, spectra were measured for mixtures of depolymerized DNA and three of the basic dyes.

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METHODS

Absorption spectra of the following dyes were studied by means of a Beckman spectrophotometer in the wave length range of 320 m μ to 700 m μ .²

2-aminoacridine ³	Coriphosphine (1-methyl-3-amino-6-dimethylaminoacridine)
3-aminoacridine ³	Rivanol (lactate of 3-amino-7-ethoxy-9-aminoacridine)
9-aminoacridine	3,6-diamino-10-methylacridinium chloride
2,8-diaminoacridine ³	Berberine sulfate
3,6-diaminoacridine	Neutral red
3,7-diaminoacridine ⁴	Phenosafranine
4,5-diaminoacridine ⁴	Rhodamine B
4,6-diaminoacridine ⁴	Rhodamine 6G
4,8-diaminoacridine ⁴	Thionine
3-amino-7-dimethylaminoacridine ⁴	
N-methylacridinium chloride	

Each dye was dissolved in one tenth molar phosphate buffer at pH 7.0 in such concentration that tendencies of the dye to polymerize were minimized and that the absorption values were conveniently measurable in cells of one centimeter light path. These concentrations varied from 0.671×10^{-5} M to 2.830×10^{-5} M. The spectra of these solutions were recorded at room temperature over that portion of the wave length range mentioned above in which the dye showed significant absorption.

Spectra of the dyes were then obtained for a comparable concentration of the dye dissolved in one tenth molar phosphate buffer at pH 7.0 containing 0.5 per cent RNA. In like manner spectra of the buffered dyes in 0.5 per cent DNA were obtained.

A series of absorption spectra were prepared for solutions of six dyes of representative chemical structure in which the pH and the concentration of the dyes were held constant and the concentration of the nucleic acids was varied from 0.5 to 5×10^{-4} per cent.

Spectra were measured of solutions of these six dyes in which the dye, buffer, and nucleic acid concentrations were held constant while the pH was varied over a range from pH 3.0 to pH 10.0.

Two solutions containing 0.064 mg/ml of gelatin, 3 mg/ml of MgSO₄, and 2.5 mg/ml DNA in tenth molar phosphate buffer at pH 7.0 were prepared. One was used to determine the absorption spectra of 3,6-diaminoacridine, 3,6-diamino-10-methylacridinium chloride, and phenosafranine in the presence of highly polymerized DNA using serial dilutions of the DNA. To the second solution was added enough crystalline desoxyribonuclease to give a concentration of 1.25 mg/ml. This solution was allowed to stand at room temperature for two days and then was used for measurement of the effect of the depolymerized acid on the same three dyes.

Preliminary experiments showed that the error caused in the absorption spectra by the fluorescence of the dyes was so small as to be negligible.

² The numbering of the acridine ring used by Chemical Abstracts is used throughout this paper.

³ Generously supplied by Dr. A. Albert, Australian National University, Canberra, Australia.

⁴ Generously supplied by Dr. A. Goldberg, Ward, Blenkinsop & Co., Ltd., Shepton Mallet, Somerset, England.

A check for *in vivo* nuclear staining was made on three of the dyes not previously available. Of these three dyes, 2-aminoacridine, 3-aminoacridine, and 3,7-diaminoacridine, only the 2-aminoacridine failed to stain nuclei strongly.

RESULTS

The position and intensity of the major absorption peaks for the entire series of dyes in buffer alone and the changes with 0.5 per cent RNA and 0.5 per cent DNA at pH 7.0 are listed in Table I. The positions of the peaks

TABLE I

Changes in the Major Absorption Peaks of Basic Dyes Plus Nucleic Acids in M/10 Phosphate Buffer at pH 7.0.

Dye	Buffer Alone		0.5 per cent RNA		0.5 per cent DNA	
	λ m μ	ϵ mol	$\Delta \lambda$ m μ	$\Delta \epsilon$ mol	$\Delta \lambda$ m μ	$\Delta \epsilon$ mol
2-aminoacridine	357	6,200	+ 3	-1,700	+ 15	- 2,500
3-aminoacridine	366	14,000	+ 4	-5,400	+ 7	- 6,400
	454	11,900	+ 16	-1,800	+ 16	- 2,700
9-aminoacridine	402	9,700	+ 6	-3,400	+ 6	- 4,800
2,8-diaminoacridine	430	3,600	+130 ¹	-1,400 ¹	+130 ¹	- 1,100 ¹
3,6-diaminoacridine	443	29,000	+ 19	- 200	+ 21	+ 100
3,7-diaminoacridine	368	9,500	+ 14	-3,000	+ 17	- 3,500
	490	8,900	+ 28	-1,500	+ 32	- 1,800
4,5-diaminoacridine	405	4,500	+ 19 ¹	-1,900 ¹	+ 30 ¹	- 3,100 ¹
4,6-diaminoacridine	400	9,300	+ 18	+3,100	+ 25	+ 2,400
4,8-diaminoacridine	405	4,800	+ 15	+ 400	+ 20	+ 400
3-amino-7-dimethylaminoacridine	367	5,900	+ 16	-1,400	+ 15	- 600
	500	5,800	+ 50	-1,100	+ 50	- 100
N-methylacridinium chloride	358	18,400	+ 2	-7,400	+ 3	- 9,800
Coriphosphine	462	5,000	+ 15	+1,200	+ 12	+ 1,200
Rivanol	362	12,200	+ 14	- 500	+ 13	- 1,700
3,6-diamino-10-methylacridinium chloride	452	38,200	+ 16	+4,600	+ 18	+ 2,400
Berberine sulfate	345	31,700	+ 6	-8,800	+ 11	-10,800
	420	7,300	+ 15	-2,300	+ 24	- 2,700
Neutral red	525 ²	25,500 ²	+ 31	-2,900	+ 29	- 2,700
Phenosafranine	523	41,300	+ 21	-3,600	+ 19	- 3,100
Safranine O	521	33,100	+ 22	+2,700	+ 20	+ 700
Rhodamine B	555	109,550	0	0	0	0
Rhodamine 6G	529	77,500	+ 4	-7,000	+ 7	- 9,500
Thionine	602	12,700	+ 15	-1,900	+ 17	- 3,300

¹ Peak very wide and diffuse.

² pH 3.0.

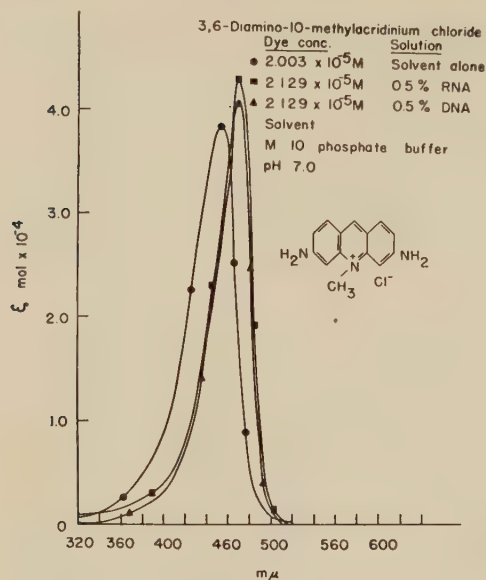


FIG. 1

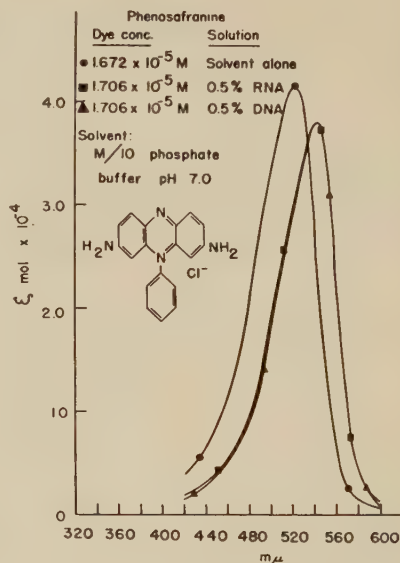


FIG. 2

are listed by the wave length in $m\mu$ of maximum absorption and absorption intensities are listed as units of ϵ mol which are values of the extinction coefficient for a one molar solution and a light path of one centimeter. This value is used as it removes the dependence of the height of the absorption curve on the concentration. Representative absorption curves are also shown in Figs. 1 and 2.

An examination of these data shows that a large number of the basic dyes show a similarity of interaction with the nucleic acids. With the exception of the rhodamine B all of the dyes tested, whether acridines or not, show shifts of the absorption peaks to longer wave lengths and in most cases there is a reduction of the intensity of absorption. In general, the mono-aminoacridines show smaller shifts in the wave length of maximum absorption than the diaminoacridines.

Table II and Figs. 3 to 6 show the effects on the spectra of a selected series of six dyes caused by varying the concentration of the nucleic acid component. In all cases the DNA is observed to have a more pronounced effect on the spectra than the RNA. This is evident from the marked shifts in the absorption peak positions which occur at concentrations of DNA which are at least 1/10 of those at which significant peak shifts occur with RNA. It is furthermore noted that major shifts of the absorption maxima of 3,6-di-

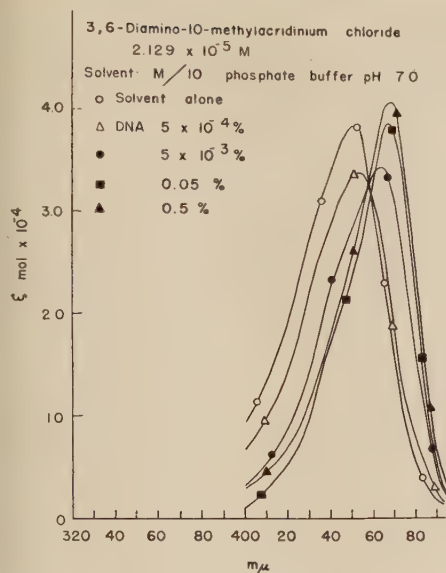


FIG. 3

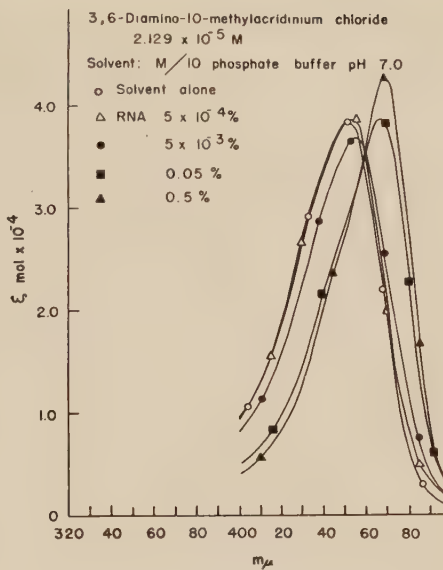


FIG. 4

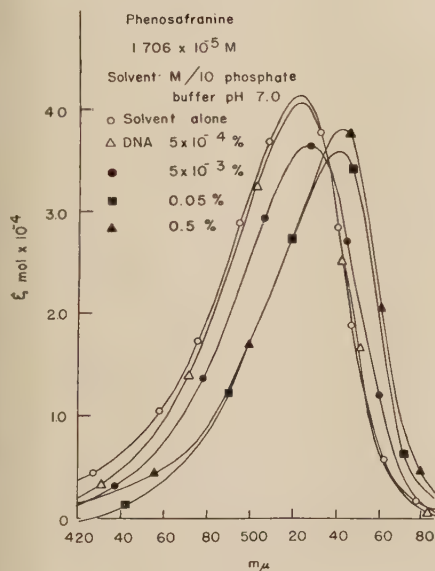


FIG. 5

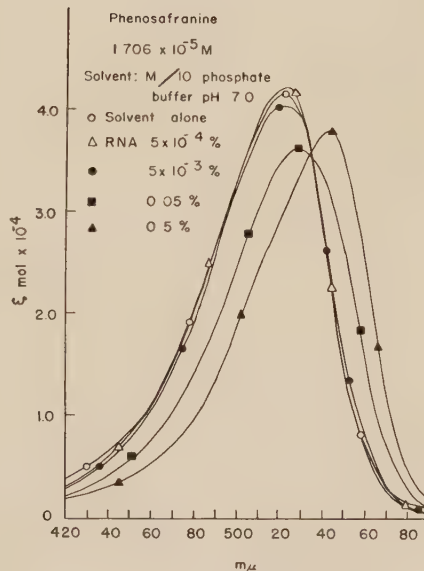


FIG. 6

aminoacridine, 3,6-diamino-10-methylacridinium chloride and thionine occur at concentrations of nucleic acid 1/10 of those at which the peak shift occurs with 9-aminoacridine and phenosafranine. Neutral red occupies

TABLE II

Changes in the Absorption Peaks of Basic Dyes with Varying Concentrations of Nucleic Acids in M/10 Phosphate Buffer at pH 7.0.

Per cent Nuc. Acid	3,6-diamino-acridine		3,6-diamino-10-methyl-acridinium chloride		9-amino-acridine		Neutral red		Phenosafranine		Thionine	
	λ m μ	ϵ mol	λ m μ	ϵ mol	λ m μ	ϵ mol	λ m μ	ϵ mol	λ m μ	ϵ mol	λ m μ	ϵ mol
RNA												
0.0	443	29,000	452	38,200	402	9,700	525	25,500 ¹	523	41,300	602	12,700
Changes:												
5×10^{-4}	+ 2	-2,200	+ 2	+ 400	0	- 800	- 3	-13,500	+ 1	+ 800	0	-1,200
.005	+ 4	-2,800	+ 3	-1,600	0	+ 400	0	-13,300	0	-1,100	+ 2	-2,000
.05	+17	-2,000	+15	+ 100	+1	-1,500	+20	- 6,800	+ 7	-5,200	+14	-2,800
.5	+19	- 200	+16	+4,600	+6	-3,400	+31	- 2,900	+21	-3,600	+15	-1,900
DNA												
0.0	443	29,000	452	38,200	402	9,700	525	25,500 ¹	523	41,300	602	12,700
Changes:												
5×10^{-4}	+ 3	0	+ 2	-4,500	-1	0	+ 1	-12,900	+ 1	- 600	0	-1,700
.005	+13	-3,000	+12	-3,900	+1	-2,700	+ 8	- 9,800	+ 5	-4,800	+ 8	-3,300
.05	+19	- 100	+16	+ 300	+6	-4,500	+25	- 3,400	+17	-5,400	+15	-2,700
.5	+21	+ 100	+18	+2,400	+6	-4,800	+29	- 2,700	+19	-3,100	+17	-3,300

an intermediate position in this respect. There is generally a decrease in the molar extinction caused by the addition of small amounts of nucleic acid. This action on the intensity of absorption is usually removed by increasing concentrations of nucleic acid.

The results of altering the pH of the mixtures are shown in Table III and in Figs. 7 to 10. In the absence of nucleic acid there is a very marked alteration in the position and intensity of absorption peaks of neutral red and 3,6-diaminoacridine as the pH is varied from acidic to strongly basic. The other dyes show no alteration of position of absorption maxima but there are only variations in intensity of absorption as the pH changes.

The very strong power of nucleic acid to maintain the acid form of the dye in strongly basic media is apparent in this experiment. The effect of added nucleic acids is especially clear in those dyes which show appreciable changes with pH in buffer alone. Neutral red exists as an equilibrium mix-

¹ Extinction coefficient at pH 3.0.

TABLE III

Changes in the Absorption Peaks of Basic Dyes Plus Nucleic Acids in M/10 Phosphate Buffer at Varying pH.

Dye	Buffer Alone		0.5 per cent RNA		0.5 per cent DNA	
	λ m μ	ϵ mol	λ m μ	ϵ mol	λ m μ	ϵ mol
pH	3.0		3.0		3.0	
3,6-diaminoacridine	446	31,100	+18	- 2,400	+17	- 1,500
3,6-diamino-10-methylacridinium chloride	454	42,800	+16	- 2,800	+14	- 1,600
9-aminoacridine	402	10,400	+ 5	- 3,300	+ 6	- 5,100
Neutral red	528	25,500	+28	+ 200	+26	+ 500
Phenosafranine	522	41,000	+16	- 4,100	+20	- 6,000
Thionine	602	12,100	+18	- 2,100	+18	- 500
pH	7.0		7.0		7.0	
3,6-diaminoacridine	443	29,000	+19	- 200	+21	+ 100
3,6-diamino-10-methylacridinium chloride	452	38,200	+16	- 200	+18	+ 2,400
9-aminoacridine	402	9,700	+ 6	- 3,400	+ 6	- 4,800
Neutral red	525	25,500 ¹	+31	- 2,900	+29	- 2,700
Phenosafranine	523	41,300	+21	- 3,600	+19	- 3,100
Thionine	602	12,700	+15	- 1,900	+17	- 3,300
pH	10.0		10.0		8.0	
3,6-diaminoacridine	400	12,000	—	—	—	—
	440	11,700	+22	+13,500	+23	+17,400
3,6-diamino-10-methylacridinium chloride	454	35,200	+14	+ 4,000	+14	+ 6,300
9-aminoacridine	403	9,400	+ 4	- 2,800	+ 5	- 4,100
Neutral red	453	11,300	+10	+ 1,100	+15	- 400
Phenosafranine	522	41,000	+17	- 2,500	+20	+ 200
Thionine	601	6,900	+19	+ 3,300	+17	+ 4,100

ture of two forms and has sufficient sensitivity to pH changes to form a useful acid-base indicator. In neutral pH, about half of the dye is in the basic form. Adding nucleic acid causes a conversion into the acid form. When the new peak is compared with that of the dye in acid solution, however, the same changes in wave length and absorption intensity observed in the other dyes, is found to have occurred. The basic form of 3,6-diaminoacridine is converted by nucleic acid into the acid form entirely and does not

¹ Extinction coefficient at pH 3.0.

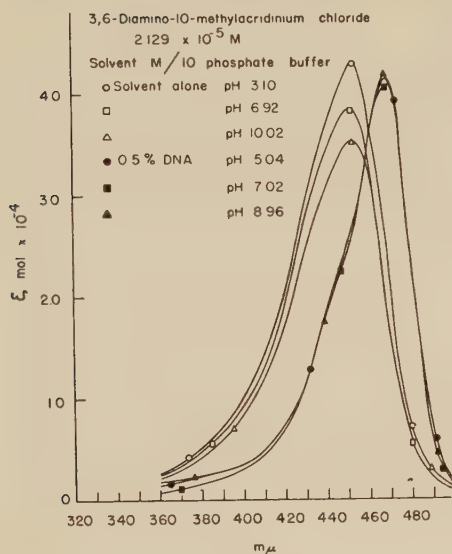


FIG. 7

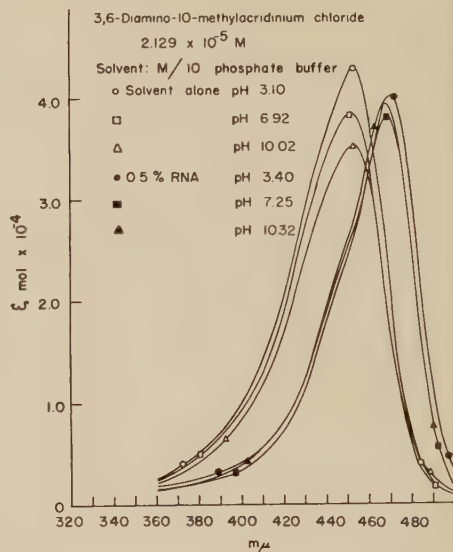


FIG. 8

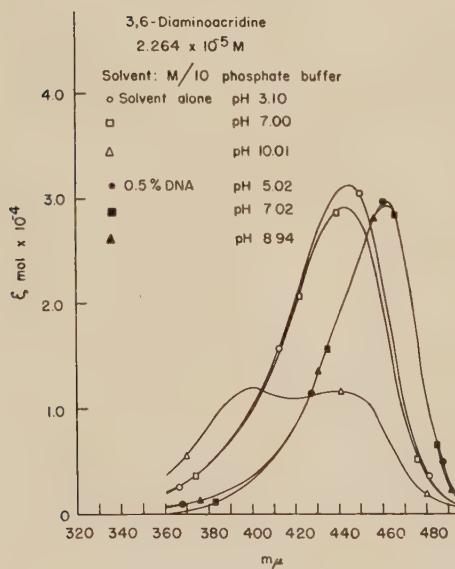


FIG. 9

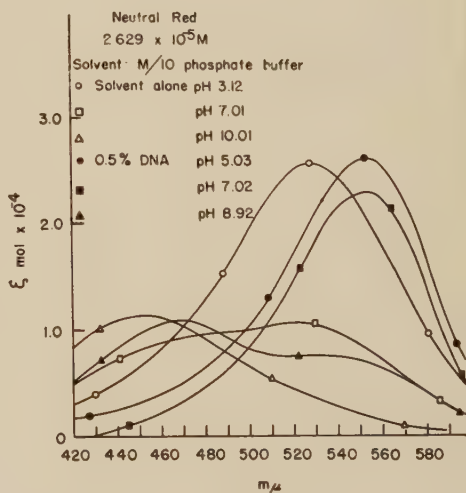


FIG. 10

occur in the spectrum. The spectra of the other dyes show that in many instances adding nucleic acid in sufficient concentration has the same effect on the molar absorption coefficient as decreasing the pH. It is further ob-

TABLE IV

Changes in the Major Absorption Peaks of Basic Dyes Plus Polymerized and Depolymerized DNA in M/10 Phosphate Buffer at pH 7.0.

Per cent Nuc. Acid	3,6-diaminoacridine		3,6-diamino-10-methyl- acridinium chloride		Phenosafranine	
	λ m μ	ϵ mol	λ m μ	ϵ mol	λ m μ	ϵ mol
Polymerized DNA						
0.0	444	25,600	454	41,600	523	42,900
Changes:						
5×10^{-4}	+ 2	- 5,700	0	- 6,400	0	- 1,600
.005	+11	- 6,300	+ 9	-11,300	+ 3	- 6,900
.05	+16	- 3,400	+14	-12,800	+17	-10,900
.25	+18	- 3,200	+15	- 4,300	+19	-10,500
Depolymerized DNA						
0.0	444	25,600	454	41,600	523	42,900
Changes:						
5×10^{-4}	0	- 800	0	- 900	0	+ 900
.005	0	- 400	0	- 900	0	- 500
.05	+ 3	-1,700	+ 1	- 2,400	+ 1	+ 1,300
.25	+ 5	-1,900	+ 7	- 4,300	+ 1	- 1,400

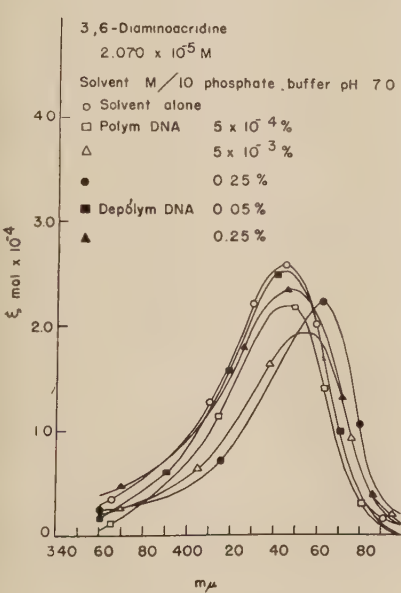


FIG. 11

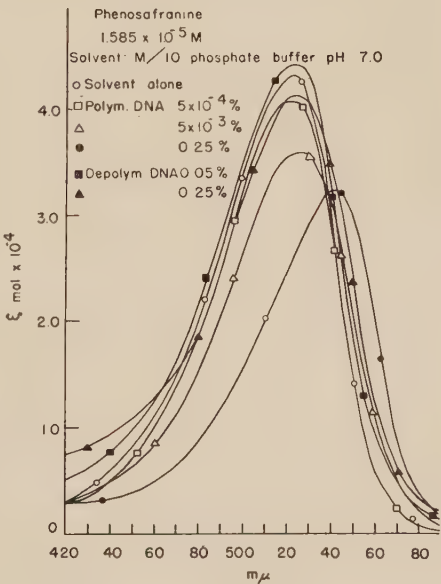


FIG. 12

served that with the exception of neutral red which presents a special case neither acid nor base cause any marked shift in the position of the maxima when the nucleic acids are present.

Table IV shows the changes in absorption spectra of mixtures of three dyes with varying concentrations of polymerized and depolymerized DNA. Selected examples of these spectra are plotted in Figs. 11 and 12.

It is noted that although a concentration of 0.25 per cent polymerized DNA causes a maximum shift in the absorption peak of all three dyes only in the case of 3,6-diamino-10-methylacridinium chloride does the depolymerized DNA cause any appreciable displacement. Further, the displacement caused by 0.25 per cent depolymerized DNA in this one case is equivalent to that caused by a concentration of 0.005 per cent for the polymerized DNA. It is thus evident that the degree of polymerization has a marked effect on the formation of the nucleic acid-dye complex in the case of DNA.

DISCUSSION

The experimental data show that a bond is formed between the nucleic acids and the dye molecules for a majority of the dyes studied. That this bond is more than a simple electrostatic attraction between ionic species is demonstrated in two ways. First, in the highly buffered medium, the spectrum is that of the salt form of the dye as is shown in the case of 3,6-diamino-10-methylacridinium chloride where decreasing the pH of the solvent in the absence of nucleic acids had no effect on the wave length of maximum absorption, but increased ϵ mol. Thus the absorption peak shift observed on addition of nucleic acid demonstrates that an interaction other than a simple ionization (as in the buffer alone) has occurred with the dye. Secondly, the slight effect on the dye-nucleic acid spectrum when the pH is changed markedly also indicates a more complex bonding for with increasing acidity the nucleic acid should be easily displaced from a simple salt-like bond resulting in a spectrum approaching that which occurs in the absence of nucleic acids.

All of the dyes showing a marked shift in absorption peaks in the presence of nucleic acid—even the non-acridine dyes—contain at least one external amino nitrogen atom in addition to a nitrogen atom incorporated in the aromatic ring system. From the group of aminoacridines studied, it appears that a certain spacing of these amino groups is also necessary for maximum interaction of the dye with the nucleic acid molecule. The addition of three new aminoacridine dyes to the list previously investigated by De Bruyn

et al. (2, 3) for *in vivo* nuclear staining ability, lends emphasis to the theory of preferential spacing of active groups. Of the dyes tested to date, it is significant that for all those found capable of staining living nuclei a common factor is the presence of an amino group attached to an acridine ring system at the number three or six position. Neither monoamino- nor diaminoacridines which lack this grouping have been found capable of staining. In the number three and six positions the amino groups are able to interchange electrons with the ring nitrogen. In this way they are capable of making contributions to the resonance stabilization of the ionic form of the molecule as a whole. In all of those structures with at least one amino group in one of the preferred positions, the absorption peaks are much more strongly defined.

Kurnick (4) proposes that the longer molecular chain in the polymerized DNA allows more sites at which two or more phosphoric acid groups are so arranged spatially as to permit an easy fitting with two or more of the basic groups of the dye molecule. This probably forms the basis for the results obtained in the present experiments with depolymerized DNA. These aminoacridine dyes present an even more rigid test of the hypothesis than do the triphenylmethane dyes used by Kurnick as the spatial requirements are more rigidly fixed by the fused ring nucleus of the dyes. From the present study it appears that the nucleotides must be coiled in such a way as to permit at least two of the basic centers to interact with the coil, fixing the dye strongly to the nucleic acid molecule.

Michaelis (5) has postulated that the dye molecules may lie in between the separate nucleotide leaves of the acids with one of the active basic groups contiguous to a free phosphate radical. This concept of the dye molecules lying between the nucleotide leaves is attractive in helping to explain the strong affinity of the nucleic acids for the flat ring systems studied here. Of the dyes studied, the ones having the greater interaction with the nucleic acids are those possessing a nearly completely planar configuration with the attached amino groups lying in the plane of the rings and with no bulky constituents lying out of this plane. Thus, these dyes require a minimum of deformation to permit them to fit in between nucleotides. Albert (1) in his study of the bacteriostatic effects of acridines has noted that hydrogenation of one of the side rings removes the bacteriostatic effect of the dye. The physical result of such treatment is merely an increase in the thickness of one side of the ring system. However, the effect of the thin ring system fitting between the nucleotides is obviously of secondary importance to the chain length factor because of the pronounced effect of depolymerization of the acid on the dye-acid complex.

The strength of the complex bond between dye and nucleic acid is apparent in the case of the two dyes which showed a major alteration in spectra with pH changes. The 3,6-diaminoacridine shows a strong tendency to polymerize in basic medium where the ionic form is neutralized and the free dye is formed. But when nucleic acid is added, this trend is completely reversed, indicating a very strong attachment. The neutral red spectra show similar effects though not to such a marked degree. Apparently the size and shape of the neutral red molecule are less favorable for close fitting to the attractive centers of the nucleic acid than are the size and shape of the 3,6-diaminoacridine.

It is notable in several cases that the DNA has a stronger effect on the dye spectra than does the RNA. This is particularly evident in the experiments in which the concentration of the nucleic acid was varied. The DNA affects the spectra at considerably lower concentrations than does the RNA. This would indicate that there are more complexing centers per gram and/or stronger bonding of the dye with the DNA. The difference in interaction of RNA and DNA with basic dyes is probably due less to the normal differences in chain length than to the degree of chain branching. Since RNA has two available sites of attachment on the sugar moiety of the nucleotide, rather highly branched molecular chains are probable with a resulting decrease in the number of sites arranged to fit the dye molecule. In the case of DNA, however, since there is only one available position on the sugar moiety to which a second nucleotide may be attached, chain branching can occur only by replacement of the third hydroxyl group of one of the phosphate groups. Thus long and essentially unbranched chains are the rule. These long and unbranched chains may then coil into various spatial configurations allowing a maximum of sites fitting the requirements of the dyes.

The differences between the effects of RNA and DNA on the dye spectra, correlate with the observation that the staining dyes show up in the nucleus of the living cell—the location of the cell's DNA. However, this latter observation fails to explain the observed staining of the nucleolus (2) by these dyes since the nucleic acid of the nucleolus is generally conceded to be of the RNA type. Nürnberger, Engström and Lindström (6) have presented data showing the concentration of RNA in the nucleolus to be approximately four times greater than its concentration in the cytoplasm. In the present work it is shown that RNA causes spectral shifts similar to those caused by DNA but at significantly higher concentrations of nucleic acid. Perhaps, then, this concentration difference for RNA between the nucleolus and the cytoplasm is the reason for the appearance of the aminoacridines in the

nucleolus but not in the cytoplasm. While the cytoplasmic concentration of RNA is greater than that found necessary to displace the absorption peak in the present study, in the living cell the dye must compete with many other substances for the favorable attachment sites of the acid.

The experiments in which the nucleic acid concentration was varied show that the spectra of the dyes capable of *in vivo* nuclear staining are affected by both types of nucleic acid at lower concentrations than are the spectra of the other basic dyes with the exception of thionine. Thus the chemical properties of the nucleic acids, especially DNA, and the amino substituted dyes containing a ring nitrogen are such that strongly bonded complexes based on complementary configurations are formed. The specificity of the *in vivo* staining by the aminoacridines may be related to this difference in the strength of the interaction between the dyes and the nucleic acids.

A part of the complex bond between the nucleic acids and the basic dyes is the electrostatic attraction between the positive ionic forms of the dyes and the negatively charged centers of the acids. The variation in basic strength of the dye molecule undoubtedly contributes to the differences in the strengths of the bonds formed with the different dyes. In accord with this, Albert (1) found that the 3,6-diamino-10-methylacridinium ion was the strongest base of the acridine dyes. This same dye was found by De Bruyn *et al.* (2) to give the most intense and persistent *in vivo* nuclear staining of any dye tested.

An alternative reason for the specificity of the aminoacridines in the *in vivo* nuclear staining reaction may involve a difference in permeability of the nuclear membrane for the various basic dyes. This argument cannot be ruled out at this time as little is known concerning the nature of the membrane. However, it is difficult to see a sound chemical basis for such a permeability difference between such similar dyes as those here tested.

SUMMARY

An investigation of the absorption spectra of various basic dyes including the aminoacridines with nucleic acids shows the formation of a dye-acid complex joined by more than simple electrostatic attractions. The complex bonding is stronger for those acridines which have been shown to have an affinity for cell nuclei *in vivo*. Evidence is present to show that the dye is more tightly bound to DNA than to RNA. The degree of polymerization of DNA has a marked effect on the formation of the dye-acid complex. Pre-

sumably depolymerization destroys sites on the nucleic acid molecule which are spatially favorable to complex formation. Reasons for the staining of RNA in the nucleolus but not of RNA in the cytoplasm are discussed.

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MITOSIS IN CULTURES OF NEWT TISSUES

I. A CRITICAL STUDY OF THE METHODS AND MATERIAL

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Soon after examination of fixed preparations had led to the first descriptions of mitosis, Flemming (9) observed the division of living chondroblasts in the gill cartilages of tadpoles. Unfortunately, in tissues taken directly from an animal, cells can be observed only when closely packed, or after more or less violent means of separation. After Harrison (12) had devised a method of cultivating animal tissues *in vitro* it became possible to examine living tissue cells which had separated themselves with no mechanical interference other than the explanation of the original piece of tissue. Burrows (4), Levi (18) and Strangeways (22) described the process of mitosis in tissue culture, and Canti (6) made the accurate recording of observations more certain by following cell division *in vitro* with ciné-photomicrography. Bělař (2) similarly photographed meiosis during the spermatogenesis of grasshoppers, but used surviving rather than cultivated tissue. By phase-contrast microscopy Fell and Hughes (8) were able to show, in ciné records, more detail in the living mitotic cell than had hitherto been described.

Amphibian tissues, having cells larger than those of most homiothermic animals, are particularly suitable for the examination of intracellular detail. Harrison (12), Holmes (13), Baitsell (1), and Danes (7) cultivated amphibian tissues *in vitro*, Holmes and Danes observing mitosis. The possibility of following the course of mitosis in living cultures of newt tissues was explored by Preston (20) and by Hughes and Preston (15), and their preliminary observations provide the point of departure for the present studies.

In the investigations to be described, amphibian tissue was cultivated *in vitro*, and mitosis was observed in the living cultures by means of phase-contrast photomicrography. The appearance of the cells was compared with that seen after fixation and staining.

Cells in tissue culture may differ in form and general appearance from the corresponding cells in uncultivated tissues, and it is therefore desirable to

know how far cytological observations on the outgrowth of tissue grown *in vitro* are generally applicable. This question is considered in Part I of the present series of papers.

METHODS

Tissue culture. Fragments of lung, heart, coracoid cartilage and other tissues from freshly killed crested newts (*Triturus cristatus* varr.) were explanted into a mixture of fowl plasma and chick embryo extract, and incubated at 26°C. By careful dissection, organs were removed aseptically and no antibacterial agents were ever added to the medium. Explants were approximately 1 mm in diameter, except the coracoid cartilages which were explanted whole, but without any attached bone or muscle. Explants of lung yielded a rapid outgrowth of fibroblasts, epithelium and macrophages; the epithelium grew in sheets similar to those described by Danes (7). Heart and the perichondrium of the coracoid were used as a source of fibroblasts and grew particularly well after replenishment by Maximow's method, described below.

The embryo-extract was prepared freshly whenever needed, 2 cc of pulped 11-day chick embryo being mixed with 1 cc of Tyrode's solution and 3 cc of distilled water. The supernatant fluid of the centrifuged mixture was then added to the medium. For the cultivation of mammalian and avian tissues the embryo extract consists only of Tyrode's solution and embryo pulp, but for newt tissues the more dilute solution, after mixture with an equal part of cockerel's plasma, provides a better culture medium than extracts of higher or lower concentration. This was shown in three ways; media of several solute concentrations were compared and it was found that: 1) the amount and mitotic activity of the outgrowth was greatest in a medium made from the extract described, 2) such a medium had a tonicity nearer to that of newt erythrocytes than had media made with extracts of higher or lower solute concentration, and 3) on comparison of vapour pressures, this medium was found to have an osmotic pressure nearer to that of newt lymph than had the other media tested; this last observation of course is only confirmatory, as tonicity rather than osmotic pressure is the important variable to be controlled. An experiment with a short series of cultures indicated no advantage in using 9- or 13-, rather than 11-day chick embryos.

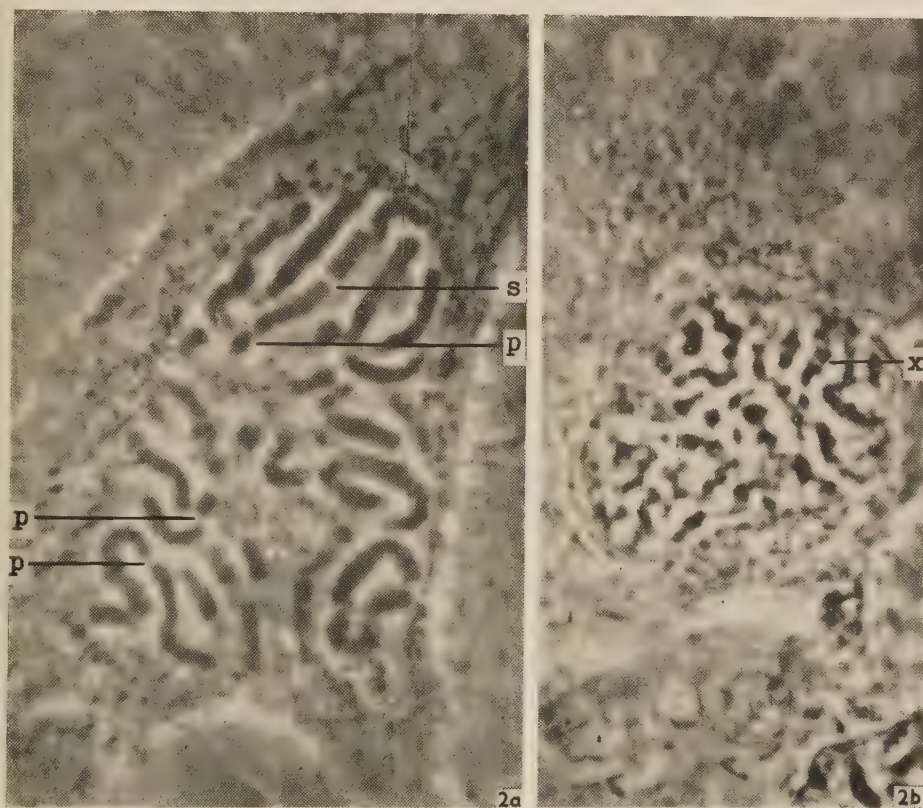
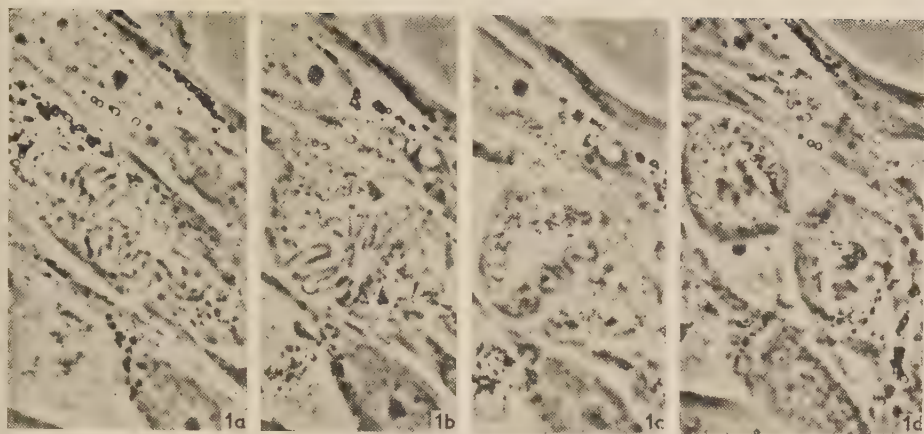
One drop of the embryo-extract was mixed on a sterile cover slip with one drop of plasma, and the tissue fragment was put into the mixture before the latter clotted. The cover slip was then inverted and sealed with molten paraffin wax on to a cavity slide.

After 4-7 days the mitotic activity in the outgrowth of a culture began to decrease

Fig. 1. Part of living culture showing cardiac fibroblast in mitosis. Phase contrast $\times 700$. a) The chromosomes are taking up their positions to form the metaphase plate. b) Metaphase plate. c) Metaphase. d) The nuclear membranes have just formed around the clumped groups of daughter chromosomes.

Time intervals: a)-b)—18½ minutes. b)-c)—22 minutes. c)-d)—22 minutes.

Fig. 2. Living perichondrial fibroblast in mitosis. Phase contrast, $\times 1,750$. a) Early metaphase, p—primary constriction; s—secondary constriction. b) Telophase of the same division 3 hrs. 22 mins. later. x—Chromosome showing longitudinally double structure with beading.



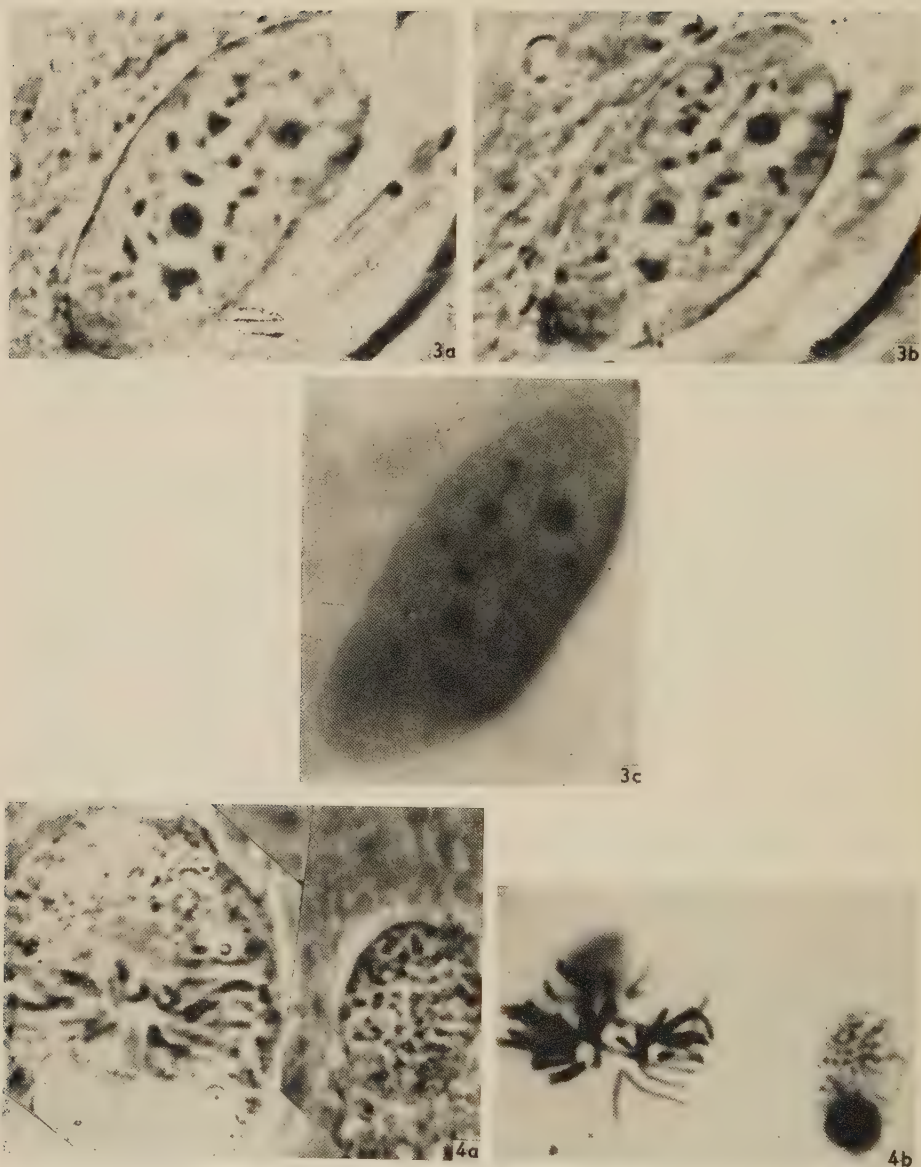


Fig. 3. Nucleus of perichondrial cell at interphase, $\times 1,600$. a) Alive. Phase-contrast. b) Same cell but photographed with microscope focussed 1μ lower. c) Same cell, fixed within one minute of being photographed alive, stained with Feulgen's reaction and light green. Except for the three nucleoli, all intranuclear detail seen in the photograph is Feulgen-positive. Shrinkage is slight and structural detail is well preserved.

Fig. 4. Cardiac fibroblasts in mitosis: metaphase plate and telophase nucleus $\times 1,500$. a) Alive.

and subcultivation became necessary. It was seldom possible to transplant a zone of the delicate outgrowth with the explant after the first passage but, even when the original fragment was subcultivated without its outgrowth, the latency before the emergence of cells was reduced at the second passage.

Extensive outgrowths were obtained by repeated replenishment of the medium by Maximow's method, which was carried out as follows (cf. Cameron (5)). A sterile round glass cover slip, 2.2 cm in diameter was attached to a mica slip 4 cm square by a small drop of sterile water or saline. On the round cover slip a hanging-drop culture was prepared, as already described; the mica cover slip with the adherent round cover glass was inverted over the well, 4 cm in diameter, of a cavity slide, $7\frac{1}{2} \times 4\frac{1}{2}$ cm, and was sealed to the slide with paraffin wax. The cultures were subcultivated by replenishment without excision. The large mica slip, after being taken off the slide, was slightly bent between the thumb and index finger, so that the edge of the small slip stood clear of the large one, allowing the former to be grasped in a forceps and lifted off vertically; in this way the small cover slip was removed without sliding it over the non-sterile edge of the mica. The small slip, bearing the culture, was placed in Tyrode's solution, diluted with one-third of its own volume of distilled water, for about twenty minutes at room temperature. The slip, taken out of the diluted Tyrode's solution, was laid on a freshly sterilized mica cover slip; since the small cover slip was already wet, no drop of water had to be added to make the slips adhere. The culture was replenished with equal amounts of embryo extract and plasma, which were mixed on a sterile slide and added, before clotting, to the culture. The mica cover slip, with the smaller glass one attached, was then sealed, as before, on to a cavity slide.

This process replaced transplantation and allowed the culture to grow undisturbed. After several passages by Maximow's method it was possible, and sometimes necessary, to transplant the tissue fragment with the zone of outgrowth, the whole culture often being divided into two, three or four daughter cultures.

The experiments recorded in this series of communications involved the preparation of rather more than one thousand cultures, most of which were subcultivated several times. By a combination of Maximow's method and transplantation, cultures were maintained for over four months and could probably have been kept growing indefinitely. The outgrowth sometimes extended for 2 mm beyond an explant 1 mm in diameter, and as many as 150 mitotic figures were found in one culture.

Observation of living cells in tissue culture. In order to observe a living culture under the most favourable conditions, the tissue was placed in a parallel-sided chamber, one side of which consisted of the cover slip bearing the culture; the vessel is similar to that used by Hughes (14). While living cells were being watched and photographed by phase-contrast microscopy, the culture under observation was kept at 26°C by a thermostatically controlled warm box enclosing the lower part

Phase-contrast. b) Same cell after fixation in Maximow's fluid, treatment by Feulgen's reaction and staining by light green. Fixation within 1 minute (metaphase) and $1\frac{1}{2}$ minutes (telophase) of photography. The Feulgen-positive mass overlying part of the telophase nucleus is a pyknotic nucleus out of focus in the photograph of the living cell. The material appearing white, on each side of the metaphase plate in (a), is fat. Note the shrinkage of the telophase nucleus (cf. Fig. 3).

of the microscope. The phase-contrast microscope itself, made by Messrs. Cooke Troughton and Simms of York, was used with a 90° phase-plate, and photographs were taken on Kodak "Microfile" film with a 35 mm reflex camera. A 48-watt 6-volt filament lamp was used throughout. Figs. 1 and 2 show examples of the results obtained.

In photography and in observing cells for long periods, it is necessary to consider whether the cells are damaged by the illumination. That electro-magnetic radiations with wave lengths shorter than those of visible light injure living tissues is well known, and in making observations by visible light it is important to ascertain what abnormalities, if any, may be caused by illumination. If cultures of newt tissues are illuminated for microscopy by light rather too bright for comfortable observation, exposures totalling more than a few seconds may cause delay or even clumping of chromosomes in metaphase followed after one or two hours by chromatolysis.

Another effect of over-illumination is the appearance of large sluggishly moving cell processes which are withdrawn only very slowly; these are different in size, shape and speed of movement from the small protrusions which are formed during normal mitosis. The larger, abnormal protrusions were not seen in the cells used for the observations presented in this series of communications.

Although cells used in the experiments recorded here were not arrested in metaphase, it was possible that they might have been abnormal in some lesser degree. Since illumination insufficient to halt mitosis may nevertheless prolong metaphase, the relative length of that stage might be expected to indicate damage by light. In 12 heart or lung cultures unexposed to light, the proportions of cells at the various stages of mitosis were as follows: prophase—19.1 per cent, metaphase—9.39 per cent, anaphase—9.3 per cent, telophase—24.5 per cent (204 mitotic figures); in a heart culture which had been partially illuminated for single-exposure photography the proportions were: prophase—27.4 per cent, metaphase—49.6 per cent, anaphase—7.1 per cent, telophase—15.9 per cent (113 mitotic figures) and in 6 cultures, each illuminated over about half its area and photographed by the 2 mm objective between 13 and 56 times before fixation, the proportions of the phases of mitosis were: prophase—24.8 per cent, metaphase—39.7 per cent, anaphase—12.9 per cent, telophase—22.6 per cent (93 mitotic figures). These figures do not suggest that the illumination used lengthened metaphase. The proportionate duration of phases in the individual living cells photographed at intervals was not readily estimated, because prophase was rarely seen in living cells when it was first detectable, and moreover the appearances by phase-contrast and those after fixation may not have indicated the formation of the nuclear membrane in telophase at quite the same stage. The proportion of metaphase-time to anaphase-time in individual photographed cells, however, did not indicate any prolongation of metaphase by the light used.

Observations of living tissues in vivo. Mitosis in the tails of newly hatched tadpoles of the Alpine newt (*Triturus alpestris*) was watched under the microscope. Each tadpole was placed in a drop of water on a glass microscope slide and a cover slip was laid on the tip of the tail. No narcotic was used, and the tadpole swam when returned to its bowl of water. The movements of the tail on the slide could be compensated by moving the microscope stage, and no difficulty was found in keeping

any one cell under observation. The 4 mm objective could be used, and the optical conditions were good enough for phase-contrast microscopy.

Histology. Samples of the organs cultivated as well as tadpoles and the regenerating tail-tips of adult newts, were fixed, embedded in paraffin wax, sectioned at a thickness of 5 μ , stained and mounted in D.P.X. Transverse sections of whole tissue cultures were similarly prepared, but usually cultures were kept on the cover slips on which they had been grown and were mounted whole. For purposes of comparison whole mounts were also made of uncultivated coracoid perichondrium, gall-bladder wall, lung teased into layers and the tail-tips of tadpoles; the tissues were fixed in a mixture of ethyl alcohol and acetic acid and stained with methyl green and pyronine, before being dehydrated, cleared and mounted in D.P.X.

The effect of fixation. The action of fixatives was studied by photographing a cell alive and then after fixation and staining. The appearances of individual cells before and after fixation have been compared in plant tissues by Martens (19) and Schaefer (21) and in cultures of animal tissues by Strangeways and Canti (23) and by Fell and Hughes (8). In the present studies the observation of Fell and Hughes that Maximow's solution (Zenker's solution without acetic acid—10 parts, 2 per cent aqueous osmium tetroxide solution—1, part neutral commercial formalin—1 part) preserved nuclear detail excellently was confirmed (Fig. 3); Maximow's fluid did not shrink or distort cells (see below). For making the achromatic figure conspicuous, Flemming's fixative was used, but this solution caused considerable cytoplasmic shrinkage. For some staining methods methanol was employed; it caused little appreciable distortion of either cell outline or nuclear structure, but its action on the smallest visible structural detail was not studied.

Uncultivated tissues were fixed in Zenker's solution for general histology, and in a mixture of alcohol and acetic acid before being stained with methyl green and pyronine.

It must be emphasised that the effect of a fixative on cells in the outgrowth of a tissue culture is not a reliable guide to the suitability of the same fixative for tissues which require considerable penetration. The virtually naked cells of a tissue culture are, moreover, fully fixed in periods too short for the preservation of pieces of solid tissue; thus a culture required only five minutes' immersion in Flemming's fluid, and two-and-a-half in Maximow's fixative.

Staining. Ehrlich's haematoxylin or Mayer's acid haemalum were used without counterstain for the general examination of cultures mounted whole. To show the general anatomy of cells, and in particular the achromatic figure, cultures were fixed in Flemming's solution and stained with Flemming's triple stain. The localisation of nucleoproteins and nucleic acids was demonstrated by a methyl green-pyronine mixture or a combination of May-Grünwald and Giemsa's stains (16).

Cultures were treated by Feulgen's reaction, after fixation in Maximow's fluid, to show the detail of chromosomes and chromonemata. Measurements were made on individual cells alive and then at various stages in the Feulgen procedure; it was found that the fixative did not affect cell dimensions nor did subsequent overnight storage in 70 per cent ethyl alcohol, but the diameter of both nucleus and cytoplasm was reduced by hydrolysis in normal hydrochloric acid at 60°C for 6 minutes. The nucleus underwent further shrinkage during dehydration in ethyl alcohol and clearing in xylene. Shrinkage was also noticeable in mitotic figures (Fig. 4).

Sections were stained with Delafield's haematoxylin and chromotrope or with methyl green and pyronine. Sections of one tadpole were stained by the Feulgen reaction.

COMPARISON OF CELLS *IN VIVO* AND *IN VITRO*

The influence of mechanical factors on cells in vitro. In most tissues of the body, cells and extracellular structures are packed together with little intervening space, while fibroblasts in the outgrowth of a tissue culture are free to spread with only the slightest restraint by neighbouring cells. It is only to be expected therefore that in intact tissues *in vivo* the cytoplasm may often be very different in shape from that in tissue cultures. The packing of cells in the body and their spread in tissue cultures may affect not only the cytoplasm but also the nucleus, where the former is not rigid. That differences arising from cultivation *in vitro*, both in the form of the cytoplasm and in the compactness of nuclear structure, are simple mechanical effects is demonstrated by a comparison of cells of different types.

The cytoplasm of fibroblasts is much less extended in compact tissues in the animal than it is in the outgrowth of tissue cultures, whereas the cytoplasm of epithelium appears scarcely altered by cultivation. Thus the apparent area of an epidermal cell in the tail-tip of a newt tadpole is similar to that of an epithelial cell in the outgrowth from an explant of lung or gall-bladder, and in cultures of the latter organ, in which every cell is distinguishable in a whole mount, there are no appreciable differences between the shape and size of cells in the explant and of those in the zone of growth. It is probable that the extended form of fibroblasts cultivated *in vitro* is due to a loosening of the normal close packing of the cells; that an extended shape is not to be regarded as an abnormality of the cell, is shown by the presence in the tail-tip of the living tadpole, of mesenchyme cells similar in form to the fibroblasts in the outgrowth of tissue cultures.

Differences between the nuclei of cells in uncultivated and cultivated tissues are similarly attributable to the close or loose packing of the cells. The nuclear structure of epithelial cells is just as compact in the outgrowth of cultures as in whole mounts of the tail-tip of the tadpole or of intact adult tissues. On the other hand, the nuclei of fibroblasts in the outgrowth are larger and less densely crowded with chromonemata than those in uncultivated tissues or in explants. That this difference between the nuclei is as much a mechanical effect as the corresponding difference in the cytoplasm, is illustrated by a comparison of cardiac and perichondrial fibroblasts in tissue culture; most of the fibroblasts in the perichondrial out-

growth contain nuclei which are more compact, and therefore more like those of uncultivated tissue, than are those of cardiac fibroblasts, but the nuclei of perichondrial cells which are flattened against the cover slip are similar in appearance to those in the outgrowth of heart cultures.

The above comparison between cells in uncultivated tissues and those in the outgrowth of tissue cultures refers to whole mounts. When sections of uncultivated tissues are compared with cultures mounted whole, the cells of the former appear very different from those of the latter, but when the cultures also are sectioned, these differences are no longer seen.

Nuclear shape and the nucleoli. As a culture ages, an increasing proportion of cells appear with numerous small nucleoli of various sizes, and irregularly shaped nuclei. Several small nucleoli of unequal size are common in nuclei of the tail-tip of the living newt tadpole. Irregularly shaped nuclei have not been seen in uncultivated tissues during the investigations to be described, but Klein (17) described lobed nuclei in the skin of the newt.

Mitosis. Abnormal mitotic figures in cultures of newt lung were about 6 per cent of the total. Estimates of the proportion of abnormal mitotic figures in uncultivated newt tissues seem not to be available, but 6 per cent is lower than the proportion of abnormal figures in human regenerating epidermis (11). That abnormal mitosis occurs in uncultivated tissues of the newt is evident from the tripolar figure depicted by Flemming (10).

DISCUSSION

The differences between cells in tissue cultures and in the corresponding tissues *in vivo*, are largely the result of mechanical factors due to the looser arrangement of cells in the outgrowth of the cultures. Cells in a culture mounted whole should not be compared with those in a section of uncultivated tissue, as cells, both *in vivo* and *in vitro*, have a different appearance when sectioned from that which they display in whole mounts. Some of the cells in older cultures differ from those normally seen *in vitro*, but they may approximate to a condition which is normal in the intact animal, where in most tissues the rate of cellular multiplication is far below that of a growing tissue culture.

There is no evidence for supposing that the results obtained by the methods described above give an incorrect picture of mitosis in the intact newt, provided always that the differences in mechanical conditions are borne in mind. Of the findings to be described in further papers of this series, some have been confirmed directly on uncultivated tissues.

SUMMARY

1. A method is described for cultivating newt tissues *in vitro* so as to provide abundant mitosis for observation during life and after fixation and staining.

2. The effects of three fixatives on individual cells have been examined.

3. The differences in appearance between cells in the outgrowth of tissue cultures and those in uncultivated tissues are most simply and consistently explained as mechanical effects of the density with which cells are packed.

Dr. Honor B. Fell, F.R.S. supervised the work reported in this series of communications. It is a pleasure to thank her, as well as all those workers at the Strangeways Laboratory whose friendly criticism and advice has contributed in many ways to this research.

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II. CHROMOSOME PAIRING IN ANAPHASE

WHILE chromosome pairing is an essential event in meiotic division, it is uncommon in mitosis. Darlington (5) refers to chromosome-pairing during mitosis in dipteran insects and polyploid plants, and states that somatic pairing, distinct from chiasma-pairing although accompanying it, may occur in meiosis.

Observations already reported briefly (1) indicate that in the newt, chromosomes pair in mitosis. This is perhaps the only recorded example of the pairing of mitotic chromosomes in a chordate, and differs from the instances cited by Darlington in two particulars: in the newt the chromosomes pair in anaphase, and their contact is so intimate that each pair has the appearance of a single chromosome. This last feature of chromosome-pairing in the newt contrasts with the "similar pair lying parallel" but not touching, in dipteran insects, and with somatic pairing in meiosis, in which "secondarily paired bivalents never 'touch' except through collapse in fixation" (5).

METHODS

Tissue cultures were prepared and observed as already described (2).

Two types of fixed material, both mounted whole, were used in this study: tissue cultures, stained by the Feulgen reaction, and tail-tips of hybrid newt tadpoles. The tail-tips, fixed in an acetic acid-ethyl alcohol mixture and stained with haemalum, were from the collection of Dr. M. Fischberg of Oxford, who very kindly put not only his preparations but also the use of his laboratory at the author's disposal.

OBSERVATIONS

During the separation of the daughter chromosomes they were sometimes seen to undergo an apparent reduction in number (Fig. 1). It is assumed that the apparently simple chromosomes of late anaphase are in fact each composed of two of the chromosomes of early anaphase. Such pairing might be expected to have the following consequences: a) during anaphase there would be an apparent decrease in the number of chromosomes; b) if pairing were between homologous chromosomes, identifiable chromosomes would appear only once in a daughter group in late anaphase; c) the late anaphase chromosomes should show some longitudinal division into their constituent halves (although, in the absence of other evidence, such fission might merely

TABLE I

Counts of chromosomes in whole mounts of newt tadpole tail-tips prepared by Dr. M. Fischberg. Heteroploidy was induced in the ova by high or low temperatures. An apparent halving of the chromosome number in anaphase is evident. (Daughter groups in the same mitotic figure are bracketed together.)

Tadpole no.	Ploidy	Metaphase	Anaphase	Telophase
1	2	20-25 20-25	10-15	
2	2	24 <i>ca.</i> 24	10-15	
3	2	22 <i>ca.</i> 24	13 11	
4	1	12 12		6 6
5 b	1	12	6 6	
6	3	36 <i>ca.</i> 33	<i>ca.</i> 19 <i>ca.</i> 20	
7	3	34	<i>ca.</i> 18 <i>ca.</i> 16	
8	24 ?3	39	<i>ca.</i> 22 <i>ca.</i> 20	
9	1	13 <i>ca.</i> 12 <i>ca.</i> 12	6 6 9 6 <i>ca.</i> 10 8	6 or 7 <i>ca.</i> 5

signify the division of a truly single chromosome into the daughter chromosomes of the next mitosis); d) the process of longitudinal adhesion should be visible in the living cell. All four expectations were fulfilled.

a) Counts made on unsquashed metaphase figures in Feulgen preparations of newt tissue cultures, confirmed that the chromosome number in the crested newt is 24. To make counts directly on anaphase groups was almost impossible in these cultures, as the central parts of the chromosomes were hardly distinguishable in the crowding at the polar end of the group. Since, however the chromosomes were all V-shaped, it was possible to



Text-fig. 1. Camera lucida drawings used for some of the counts recorded in Table I. Left-hand column — metaphase. Right-hand column — anaphase. Mitotic figures from the same tadpole are on the same horizontal line.

estimate their number by counting their free ends, all of which could be distinguished. The number of chromosomal ends trailing from an anaphase group was always about 24; since the chromosomes were V-shaped, this indicated that there were about 12 chromosomes in each group, although metaphase plates in the same cultures contained 24.

In order to count anaphase chromosomes, attempts were made to prepare squashes of tissue cultures. Squashing was made difficult not only by the coherence of the chromosomes within each anaphase group, but also by the clot of culture medium, which formed a soft matrix in which cells slipped bodily when pressed (newt tissues grow poorly in a fluid medium). The only successfully squashed anaphase group contained 12 bodies which might have been taken for single chromosomes, but each showed longitudinal splitting. There were 24 chromosomes in a metaphase plate of the same culture.

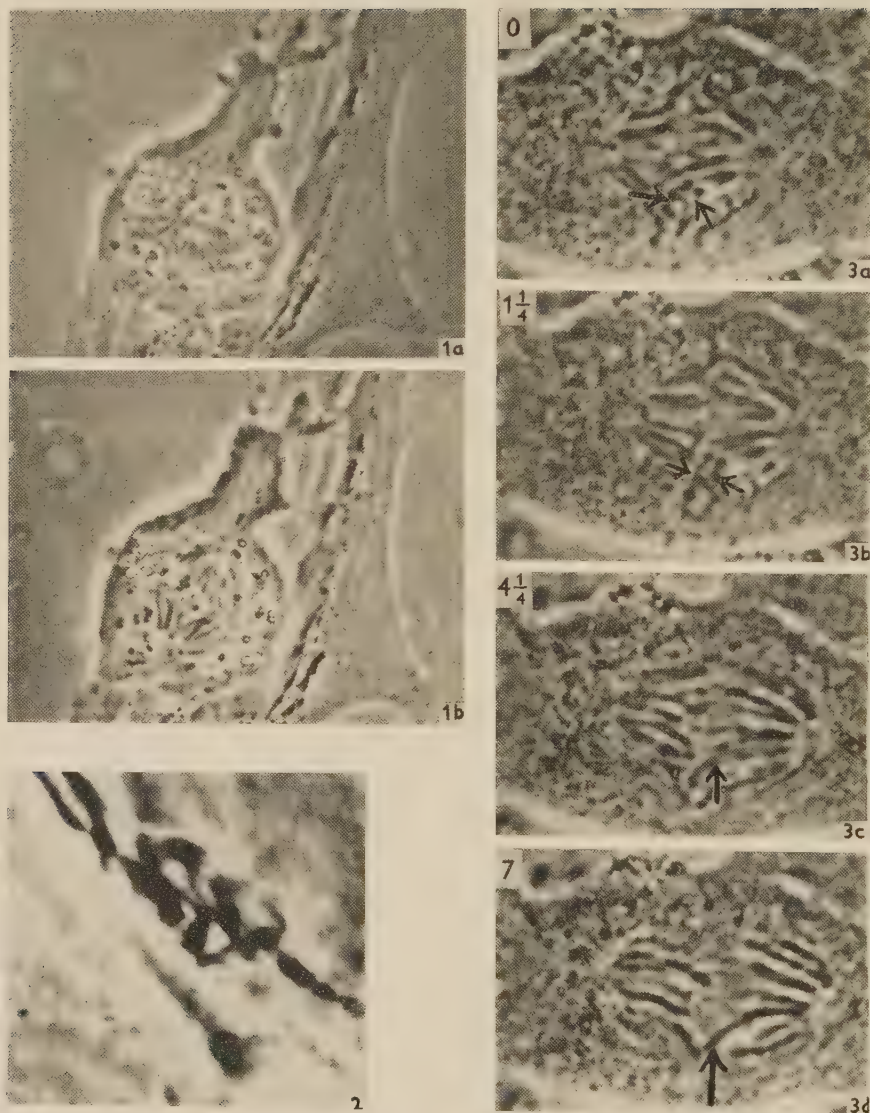


Fig. 1. Living cardiac fibroblast. Early anaphase. (a)–(b) — 5 minutes. There is an apparent decrease in number of the chromosomes. Phase-contrast. $\times 800$.
 Fig. 2. Part of an anaphase group partially disrupted by squashing in a 45 per cent aqueous solution of acetic acid. The chromosomes appear double-stranded. Phase-contrast. $\times 2,500$.
 Fig. 3. Living cardiac fibroblast, showing successive stages in anaphase. The photographs were taken from a series made at quarter-minute intervals, and on each picture is shown the number of minutes after the first exposure. a) The arrows indicate two chromosomes, the lower of which is a little out of focus. b) The two chromosomes are seen parallel to each other. c) There is now an

In tadpole tail-tips chromosomes are well displayed and may often be counted directly without squashing, even in anaphase. The chromosomes of 16 metaphase plates and 22 anaphase or telophase groups were counted; the results illustrated by Text-fig. 1, are set out in Table I, which shows an approximate halving of the apparent number of chromosomes in anaphase and telophase. That this halving was more or less exact in both haploid and triploid tadpoles shows that the chromosomes cannot always have been homologously paired, although they may have been so in diploid cells, as is shown below.

b) As stated above, if homologous chromosomes pair, any distinguishable member of a haploid set will be seen to occur only once in each diploid anaphase group. In the crested newt there is one pair of chromosomes with a secondary constriction, and, in late anaphase, this satellite-bearing chromosome was never observed more than once in a diploid group. This suggests that some pairing in anaphase was between homologous chromosomes.

c) As described above, anaphase chromosomes in a squash preparation showed a longitudinally double structure (Fig. 2). Living chromosomes sometimes appeared similarly divided, which, in view of the other evidence mentioned, may be ascribed to their having been seen during the act of pairing, although other workers, such as Creighton (4), have interpreted the double structure of anaphase chromosomes as a result of splitting rather than of pairing.

d) Continuous observations on living cells provide further evidence that the chromosomes pair during anaphase. Fig. 3 shows two chromosomes uniting to give a single body which looks like one chromosome. In the photographs it can be seen that complete fusion followed disengagement of the chromosome ends from those of the opposite group; if pairing were usually timed in this way, the infrequency with which the process could be watched clearly in living cells would be explained, for the disengagement of chromosome ends from those of the opposite group took place at a stage when in lateral view there was still great foreshortening of the chromosomes, while in polar view the two groups would be superimposed.

These findings indicate that in the fibroblasts of newt tissues anaphase chromosomes adhere longitudinally in pairs, so that each pair is seen as a single chromosome. How the paired chromosomes are bound together is not yet clear.

apparently single chromosome with a partial split. d) Finally there seems to be only a single structure. By tracing the labelled chromosomes through the whole series taken at quarter-minute intervals, their identity from one photograph to the next was established beyond doubt.

When a living fibroblast in anaphase was treated by hypotonic saline to separate the chromosomes (9) and then fixed, each of the two daughter groups showed quite clearly the diploid number of 24 chromosomes, which were thinner than the bodies which appear to be single chromosomes in similar preparations not treated with hypotonic saline. This observation indicates that the pairing can be reversed by a treatment from which living cells may, according to Hughes, recover.

Preliminary observations indicate that the paired chromosomes separate in telophase; this point is being investigated further.

DISCUSSION

The various changes which chromosomes undergo in anaphase all point to the conclusion that at some time during the separation of the anaphase groups, chromosomes come to lie in longitudinally apposed pairs, the cohesion within each pair being so close that it looks like a single chromosome. Such cohesion had been suspected by Flemming (8) who, however, later wrote "Die früher angenommene Längverschmelzung von je zwei Schleifen in den Tochtersternform habe ich nach eigenen längeren Zweifeln, besonders aber jetzt auf Grund der Arbeit von Retzius aufgegeben"—"Having long had doubts, I have now given up the previously assumed longways fusion of each two loops (chromosomes) in the daughter aster (anaphase chromosome group), especially in view of the work of Retzius". Fischberg (7) noticed that in whole mounts of the tail-tips of newt tadpoles, there was an apparent reduction of the chromosome number in anaphase; as stated above, Dr. Fischberg's own preparations have been used to confirm this observation.

Darlington (5), reviewing the work done on the pairing of chromosomes, concludes that there is a specific attraction between corresponding regions of homologous chromosomes. The mechanism of the attraction is unknown. The chromosome counts (Table I) for triploid and haploid cells, especially the latter, suggest that pairing may occur between non-homologous chromosomes. The pairing of meiotic bivalents with each other as described by Darlington must also be non-homologous.

If chromosomes pair in anaphase, it is necessary to reassess some previous descriptions of a longitudinally double structure in anaphase and telophase chromosomes. Koerperich (12) described the splitting of chromosomes during nuclear reconstruction in plants, Creighton (4) found that each anaphase chromosome of the axolotl *Amblystoma tigrinum* consisted of a double thread, and Boss (2) has shown the double structure of telophase chromo-

somes in living fibroblasts of the newt. The "vesiculation" of telophase chromosomes, seen by Kater (10, 11) in cells of the frog and the rat, and common in plants after fixation in a mixture of ethyl alcohol and acetic acid, is probably also partial longitudinal division. Apparent splits in anaphase and telophase chromosomes have usually been interpreted as divisions preparatory for the next mitosis, but the results described above indicate that these may not represent a true splitting, but a re-separation of chromosomes which have paired. No case of longitudinal division of anaphase or telophase chromosomes can be interpreted with certainty until it is known whether pairing has taken place.

The evidence given here does not justify the assumption that all chromosomes in every newt cell always pair in anaphase. Cells other than those of the newt have not yet been studied.

SUMMARY

1. In living fibroblasts in cultures of newt tissues there was an apparent reduction in the number of chromosomes in each daughter group during anaphase.

2. In stained cultures of newt tissues and in the tail-tips of newt tadpoles, the groups of daughter chromosomes in anaphase showed approximately half the expected number of chromosomes, whether the tissues were haploid, diploid, triploid or tetraploid.

3. Living fibroblasts in tissue culture were watched and photographed at frequent intervals during anaphase, and the apparent longitudinal fusion of chromosomes was recorded.

4. Pairing may sometimes have taken place between homologous chromosomes, but there must also have been non-homologous pairing.

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THE PRODUCTION OF STAINABLE GRANULES IN BACTERIA BY THE ADSORPTION OF DNA UPON THE CELL ENVELOPES

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DESPITE a substantial measure of agreement upon the main features of the bacterial cell, little real information is available upon the identity of those small granules, variously described as 'volutin' or 'metachromatic granules'. Some of these appearances, concerned with the growth of the cell envelopes, have been reviewed recently (3, 4) and further information has been added by the work of Bergersen (1, 2).

It has been suggested (4) that the basophilic material, in which the bacterial cell is exceptionally rich, may be transferred from one structure to another, by the effect of fixing and staining procedures. It has been shown (7, 8, 11) that remarkable differences in the size and appearance of the nucleus result from different fixation procedures, and there is presumptive evidence (4) that such transfer takes place between the nucleus, cytoplasm and cell envelopes in bacteria as it has also been claimed to do in plant cells (6). These observations are explained in Text-figs. 1-3. It is also known that the nucleic acid complex responsible for Gram-staining, when removed from the cell, can be adsorbed again upon the cytoskeleton under appropriate conditions (9, 10).

In this paper, evidence is advanced to show that, in bacteria which normally produce such granules, similar appearances can be produced by the adsorption of DNA of extraneous origin. This observation strongly supports the concept that such granules may arise by the local transfer of basophilic material under ordinary conditions of fixation and staining.

MATERIAL AND METHODS

A purified sample of DNA from herring roe was provided by the courtesy of Professor M. Stacey. This was used in the form of a 1 per cent solution, by weight, in physiological saline. At this concentration the solution was distinctly viscous.

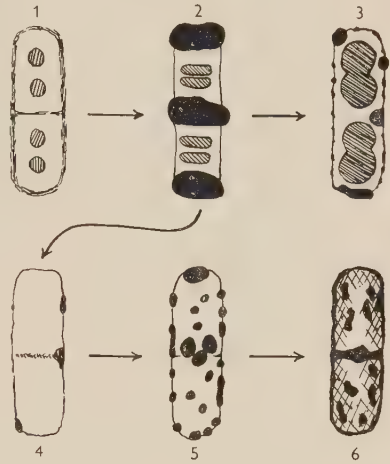
Observations were made upon one strain each of *Bacillus cereus*, *B. cereus* var. *mycoides*, *B. megaterium*, *Aerobacter aerogenes* and *Staphylococcus albus*. Cultures were grown upon meat-infusion agar at 37°C for 3–4 hours. Smears were made upon cover slips. A loopful of tap-water was placed on each smear and a loopful of DNA solution was added to it and gently agitated. In the controls, the DNA was omitted.

After being permitted to dry for a few minutes in the air at room-temperature, to cause them to adhere to the cover slips, the smears were hydrolysed for 30 mins.

Transfer of basophilic material, including DNA, under the influence of cytological procedures, in *Bacillus cereus*.

Textfig. 1–3. (Drawn from the photomicrographs, Bisset (4)), show the apparent transfer of basophilic material, firstly from the surface cytoplasm to the cross-walls and secondly, to the nucleus. In Textfig. 1 the material is slightly hydrolysed and unfixed. Stained with Giemsa, the nuclei appear small and the surface cytoplasm, basophilic. In Textfig. 2 the hydrolysis has proceeded further; the nuclei have their characteristic form as paired rods, and there is a relatively enormous aggregation of stainable material on the cross-walls. In Textfig. 3, similar material has been fixed in alcohol. The apparent size of the nuclei is much increased and granules have appeared in the cell envelopes (cf. also (11, 8)).

Textfigs. 4–6. (Drawn from the observations presented in this paper) show the adsorption of DNA upon bacilli at a further stage of hydrolysis. In Textfig. 4, hydrolysis has proceeded beyond that shown in Textfig. 2. The nuclei have disappeared and the cell envelopes are marked by a few granules. Material treated in the same way, but with the addition of DNA, shows numerous granules, especially at the tips and cross-walls (Textfig. 5) and eventually increased basophilia of the entire cell envelopes (Textfig. 6).



in N/1 HCl at 60°C, and stained with 10 per cent Giemsa solution for ten minutes (the Piekarski-Robinow modification of the Feulgen reaction). This degree of hydrolysis had previously been determined to be sufficient to remove almost all the staining capacity of the untreated bacteria, and was considerably in excess of that required to provide an adequate demonstration of their cytology by this method.

Fixation procedures, as described in the text, were also tested.

OBSERVATIONS

In the case of the strains of *B. cereus* and *B. mycoides* the controls were pale pink in colour, with a few granules. Occasional cross-walls were faintly stained, but most appeared as unstained gaps (Figs. 1 and 2, unstained cross-wall marked *a*). The test smears, to which DNA had been added, exhibited a variety of appearances. The bacteria were more darkly stained and the granules more numerous (Figs. 3 and 4). In some the cross-walls



appeared as dark bars (Figs. 5 and 6), and in others were marked by clusters of large granules (compare *a* in Figs. 1, 3, 4 and 6).

It is apparent that these conditions represent stages in the aggregation of DNA upon the cross-walls. The smaller granules represent adsorption of DNA upon other parts of the cell-envelopes and surface cytoplasm. The extent to which extraneous DNA was adsorbable by each bacterial strain was indicated by the capacity for granule formation in the controls. The *B. cereus* and *B. mycoides* strains, which showed most granules in the controls, showed maximum adsorption in the tests. In the *Staphylococcus*, which was non-granular, the difference between tests and controls was mainly one of colour; the tests appeared more purple and, in general, resembled inadequately-hydrolysed specimens, whereas the controls were manifestly over-hydrolysed. This probably represents a more even adsorption of DNA. The *B. megaterium* was intermediate in this respect, and the Gram-negative *Aerobacter* apparently failed entirely to adsorb the DNA (cf. (9)). There are considerable differences in structure, behaviour and composition between the cell envelopes of Gram-positive and Gram-negative bacteria, and these have been reviewed recently (4).

The nuclear structures were not recognisable in any of these preparations, and it is presumed that their nucleic acids were dissipated by the prolonged hydrolysis and were, in fact, represented by some of the granules in question, which must have been derived, like those of the controls, from intrinsic materials, presumably also at least partially DNA, transferred from the nucleus to the cell envelopes.

The effect of fixation of the smears, prior to hydrolysis, by immersion for five minutes in 75 per cent ethanol, 2 per cent formaldehyde or 0.05 per cent mercuric chloride was examined. These fixatives were slightly inhib-

Bacillus cereus, stained by Piekarski-Robinow modification of Feulgen reaction, with 30 min. hydrolysis in $\frac{N}{1}$ HCl, to remove the basophilia of the main nuclear and cytoplasmic elements. $\times 3,000$.

Figs. 1, 2. Controls, without added DNA. Occasional granules in cell envelopes. The cross-walls (*a*) unstained gaps or very faintly stained.

Figs. 3, 4. With added DNA, numerous granules, especially at cell-tips (growing-points) and cross-walls (*a*). Such appearances have been described by various authorities as 'volutin granules' and are among those which have been claimed to represent mitochondria and mitotic figures. The resemblance of the granules aggregated upon the cross-wall (*a*) in Fig. 4 to a group of chromosomes, complete with spindle, is in fact very close indeed. But comparison with the parallel preparations presented here reveals its true nature, as a technical artefact.

Figs. 5, 6. Greater adsorption of DNA. The cross-walls (*a*) show as dark bars, the basophilia of the cell envelopes is much augmented.

itory to the reaction; presumably because the DNA was partially destroyed before becoming fully adsorbed upon the bacilli. In the case of ethanol, an additional effect was that the granules appeared aggregated into larger masses on the surface of the bacilli.

DISCUSSION

It is apparent from the evidence presented here that granular appearances may be produced in bacteria by the adsorption of DNA. This strongly supports the suggestion (4) that transfer of basophilic material may occur between different structures in bacterial cells, as it does in plant cells (6). Some aspects of this concept are explained diagrammatically in Text-figs. 1-6.

Most of the granules produced by this method appear to have been formed in the cell envelopes and surface cytoplasm. The cross-walls and septa (presumably the basophilic elements in these) exhibit a notable affinity for DNA. The degree to which bacteria may be internally subdivided by partitions of this type is still not widely understood (cf. (5)), and it is thus a commonplace of bacterial cytology for stainable aggregations in the region of these structures to be described as intracellular granules, whereas they pertain, in fact, to the cell boundaries (3).

A further inference which must be drawn from these observations is that considerable caution must be exercised in the interpretation of stainable aggregates in any part of the cell, both as regards their form and also their cytochemical reactions, since some part of their substance may consist of displaced materials. In the latter connection, the observation of Weibull (12), who criticises certain interpretations of such granules on the ground that the reagents employed may themselves similarly aggregate in the form of granules, underlines the need for caution.

The manner in which, for example, the cluster of granules on the cross-wall (α in Fig. 4) simulates a group of chromosomes is quite remarkable. Other simulacra might be discussed *ad nauseam*, and it is pertinent to speculate to what extent this and allied phenomena may be responsible for controversial cytological and cytochemical findings in other organisms than bacteria.

SUMMARY

In bacteria which exhibit a tendency to the formation of 'cytoplasmic granules', similar granules can be produced artificially by the adsorption of DNA of extraneous origin and staining with a modified Feulgen reaction.

These granules appear mainly in the surface cytoplasm and cell envelopes, especially in the septa, and may simulate true structures.

This phenomenon supports the suggestion that differences in nuclear and cytoplasmic structures in bacteria subjected to various cytological procedures may be due to transfer of basophilic materials between different parts of the cell. It also emphasises the need for extreme caution in interpreting such minute granular structures, in respect both of their morphology and cytochemistry.

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THE CORRELATION OF GROWTH WITH INCORPORATION OF RADIOACTIVE METABOLITES INTO NUCLEIC ACIDS IN EMBRYONIC TISSUE CULTURE¹

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ONE of the best chemical indices of growth in tissue cultures is the increase in desoxyribose nucleic acid (DNA) content. Attempts to employ radioactive phosphate incorporation into nucleic acids as a convenient criterion have not been successful, because of complicating turnover processes (9, 11).

The first paper of the present series (13) dealt with the isolation of pure DNA and RNA (ribose nucleic acid) from cultures of embryonic chick hearts. It was shown that C¹⁴-labeled adenine was rapidly taken up from the nutrient medium, and that the C¹⁴ appeared in adenine and guanine of both tissue RNA and DNA.

The present study is primarily based upon the observation that radioactive adenine is incorporated only into *actively growing* heart fibroblast cultures. This incorporation has been correlated with nucleic acid synthesis for a wide range of environmental conditions. Some comparable results are also reported with isotopic thymidine.

MATERIALS AND METHODS

The source, specific activity, and purity of the adenine-8-C¹⁴ employed have been described (13).² Thymidine-2-C¹⁴ was enzymatically synthesized from thymine-2-C¹⁴ and desoxyribose-1-phosphate by Dr. M. E. Friedkin at Washington University, St. Louis, who supplied us with this nucleoside. Its activity was 1.0 mc per mM.

Highly purified growth hormone was kindly supplied by Dr. C. H. Li, University of California, Berkeley, and by Dr. S. L. Steelman, Armour Laboratories, Chicago. This material was sterilized by passage through a UF sintered glass filter.

The preparation of embryonic extract (EE) medium from fertile eggs, and the explantation and cultivation of 13-day chick heart fragments, have been described in an earlier paper (6). The nucleoprotein fraction separated from the cultures was

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² Allocated by the U.S. Atomic Energy Commission, Oak Ridge Tennessee.

analyzed for DNA by the diphenylamine reaction for desoxypentose (4), and for RNA by the orcinol colorimetric method for pentose sugars (14). The carrier technique was used for the isolation of pure DNA and RNA for radioactivity assay (13). The individual points on the curves of the figures each represent analyses on the tissue of five pooled culture tubes. For the starch column chromatographic isolation of thymine, DNA and RNA were hydrolyzed with 88 per cent formic acid (15).

RESULTS AND DISCUSSION

A marked initial fall in the nucleic acid content of chick heart fibroblasts due to extensive cellular damage during explantation has been reported (9, 3). Subsequently, large increases in the DNA and RNA levels occur (2, 10). Comparable results are shown in the curves in Fig. 1. When a combination of growth hormone and cortisone was included in the EE medium, the 6-day DNA and RNA values were increased by 8 and 12 per cent, respectively, above the control values. Somewhat greater stimulations, 28 and 30 per cent, were obtained with growth hormone plus insulin. With all three hormones, the DNA content at 6 days was 35 per cent greater than that of the control tubes. These stimulatory effects of hormones are comparable to those reported by Leslie (12). Apparently insulin is the most active component. Presumably it promoted carbohydrate utilization in the tissue cells, thereby stimulating such key processes as protein and nucleic acid synthesis.

In Fig. 2, it is significant that rapid uptake of adenine-C¹⁴ commenced during the initial phase of cultivation, when the net nucleic acid content of the cultures was actually decreasing. This early phase of adenine incorporation suggests that active nucleic acid synthesis was in progress, although extensive degradation occurred simultaneously.

It may be seen that hormones increased the adenine incorporation process. Cortisone alone had no significant effect, while growth hormone at 6 days caused a 14 per cent stimulation. A combination of the two gave a 24 per cent increase in adenine uptake. Insulin alone exerted a 46 per cent increase, while with insulin plus growth hormone or plus cortisone, the rise was approximately 70 per cent. The most marked acceleration of adenine incorporation, about 100 per cent increase, occurred when all three hormones were employed in the medium.

Friedkin (7) has shown that 23 per cent of the isotopic thymidine injected into fertile hens eggs was utilized for nucleic acid synthesis by the embryo. The label appeared only in thymine of DNA. Free thymine itself was poorly utilized. These observations have been confirmed by the present authors. It was of interest to extend the use of labeled thymidine to tissue culture.

The radioactivity of the nucleoprotein in Table I represents an uptake of approximately 3 per cent of the total thymidine-C¹⁴ employed. This lower efficiency probably reflects the very different conditions in the tissue culture experiments. The increasing specific activity in proceeding from nucleoprotein to mixed nucleic acids to DNA suggests that all of the C¹⁴ was in the DNA component. The observed absence of C¹⁴ in RNA confirmed this opin-

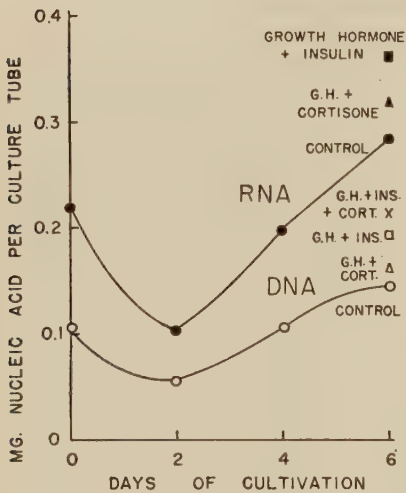


Fig. 1.

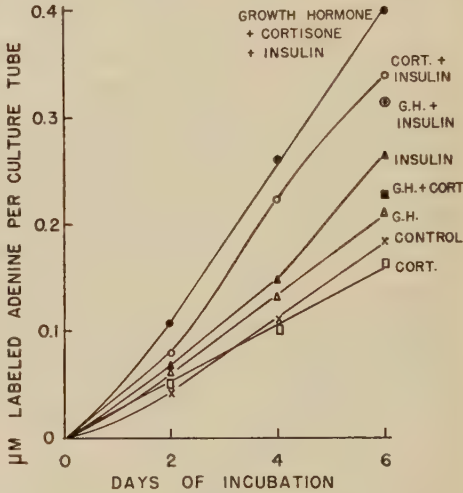


Fig. 2.

Fig. 1. Effect of hormones on tissue growth, as measured by nucleic acid content of heart cultures. The concentrations of growth hormone, cortisone and insulin were 2.5 mg, 2.5 mg, and 200 units, per 100 ml of 25 per cent EE, respectively.

Fig. 2. Effect of hormones on the rate of incorporation of adenine-8-C¹⁴ into mixed nucleic acid of cultures. The hormone concentrations were the same as in Fig. 1. The adenine concentration was 0.05 mM.

TABLE I

Influence of hormones on the incorporation of thymidine -2-C¹⁴ into DNA of heart cultures. One mg of labeled thymidine (4.1 μc) were added per 100 ml of 25 per cent EE. The cultivation period was 6 days.

Hormones added to medium	No. of culture tubes pooled	μM thymine incorporated per gm isolated material		
		Nucleo-protein	Mixed sodium nucleates	DNA
None (control)	11	1.25	5.38	10.3
Insulin + cortisone	21	2.10		
Insulin + cortisone + growth hormone	4	2.35		

ion. Upon chromatographic analysis of the DNA, virtually all of the C^{14} was found in the thymine peak.

It may be noted that insulin plus cortisone caused a 68 per cent stimulation of thymidine-2- C^{14} uptake by nucleoprotein, while with insulin plus cortisone plus growth hormone, the increase was 88 per cent. These effects are of the same order as those in Fig. 2 with labeled adenine.

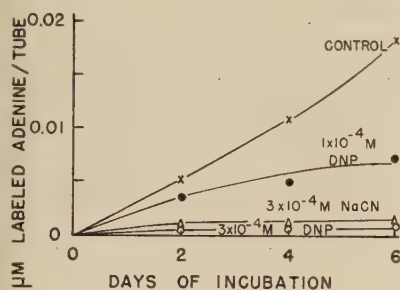


Fig. 3.

Fig. 3. Influence of respiratory inhibitors on the incorporation of adenine-8- C^{14} into mixed nucleic acids of cultures. The adenine concentration was 0.05 mM.

Fig. 4. Relative rates of incorporation of C^{14} -metabolites into mixed nucleic acids of cultures, with different nutritional media. Each labeled compound was employed at a 0.1 mM concentration.

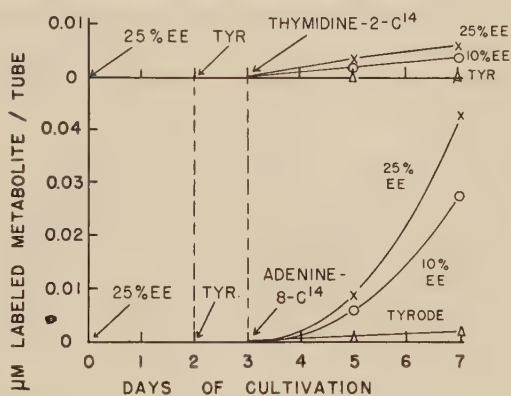


Fig. 4.

It was next of interest to test the effects of growth inhibitors on the incorporation of labeled metabolites. It has been shown that both growth and nucleic acid synthesis in tissue culture were abolished by such respiratory poisons as cyanide, azide, and dinitrophenol (5, 8). In Fig. 3, it may be seen that no significant uptake of isotopic adenine occurred in the presence of 3×10^{-4} M, NaCN or 2,4-dinitrophenol.

In seeking to correlate growth with labeled purine or pyrimidine incorporation, it was important to distinguish between nucleic acid synthesis and turnover. An approach to this problem is shown in Fig. 4. Culture tubes were maintained in an optimal medium (25 per cent EE) for an initial period of two days. Then the EE medium was replaced with Tyrode solution for one day to deplete the tissues of nutrients and to slow down cellular proliferation. At the third day the tubes in each series were divided into three groups. One group was maintained in 25 per cent EE, the second in 10 per cent EE, and the third in Tyrode solution, for two to four additional days. Each medium contained the same concentration of C^{14} -labeled adenine

or thymidine. It had been shown earlier (9) that the growth rate of heart fibroblasts was decreased by about 50 per cent in 10 per cent EE, as compared to 25 per cent EE, and that no growth, but rather progressive autolysis, occurred in Tyrode solution.

The lower part of Fig. 4 indicates that incorporation of adenine-8- C^{14} into nucleic acids was reduced in a suboptimal medium (10 per cent EE), and virtually abolished when cultures were kept in Tyrode solution. Similar results were obtained with thymidine-2- C^{14} (upper part), except that the degrees of incorporation were much lower. However, it will be recalled that, unlike adenine, thymidine gives rise to labeling in only one component of one type of nucleic acid. While adenine-8- C^{14} is less sharply localized than thymidine- C^{14} , the much greater efficiency of utilization of the former substance favors its routine application in various growth studies *in vitro*.

The absence of adenine turnover in non-growing cultures differs from results with intact adult animals, in which labeled adenine is readily incorporated into RNA of normal tissues (1).

SUMMARY

A combination of insulin, cortisone, and growth hormone stimulated the synthesis of both ribose- and deoxyribosenucleic acids of chick heart cultures, when added to the embryonic extract nutrient medium. These hormones also increased the rates of uptake of C^{14} -labeled adenine and thymidine into tissue nucleic acids. The incorporation of these compounds was correlated with the growth of the cultures: the C^{14} -uptake was reduced with sub-optimal media, and abolished when the tissue was maintained in a Tyrode solution, in which no growth occurred.

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INTERFEROMETRIC AND X-RAY ABSORPTION STUDIES OF BONE TISSUE

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BONE tissue in the adult vertebrate organism undergoes a continuous rebuilding which is rapid in spongy bone tissue but also takes place in the compact bone. The process of continuous deposition and resorption of bone goes on throughout life in most vertebrate species. The rate of the rebuilding of bone is, to a certain extent, dependent upon mechanical factors. However, the continuous fixation and release of organic and inorganic substances also takes place in bone tissue which does not have any mechanical load (1, 2). This latter process may be of great physiological importance in the regulation of certain ions in the organism.

The distribution of mineral salts in the various structures of bone tissue has earlier been investigated with quantitative X-ray microradiography. Another method for determining the masses of biological structures has recently been proposed (3, 5, 6). This method relies on measurements of the optical path difference (o.p.d.) which can be made by interference microscopy, and distributions of mass in biological material have been determined (7) by means of the Dyson interference microscope (8). This development now makes it possible to study further the processes of resorption and deposition in bone tissue.

It is the purpose of the present communication to compare the composition of identical bone structures determined by X-ray microradiography and interference microscopy.

THEORETICAL CONSIDERATIONS

Microradiography. Soft X-rays must be used to demonstrate the distribution of mineral salts in very thin sections of bone tissues. As intact bone tissue roughly consists of one quarter organic and three quarters of inorganic material (by weight) the influence of these two fractions on the total X-ray absorption has to be examined first.

The absorption of X-rays in bone tissue can be expressed as

$$I = I_o \cdot e^{-\left(\frac{\mu_i}{\rho_i} \cdot m_i + \frac{\mu_o}{\rho_o} \cdot m_o\right)} \dots\dots\dots 1)$$

where I_o and I are the intensities of the incident and transmitted X-rays respectively; $\frac{\mu}{\rho}$ is the mass absorption coefficient, where μ is the linear ab-

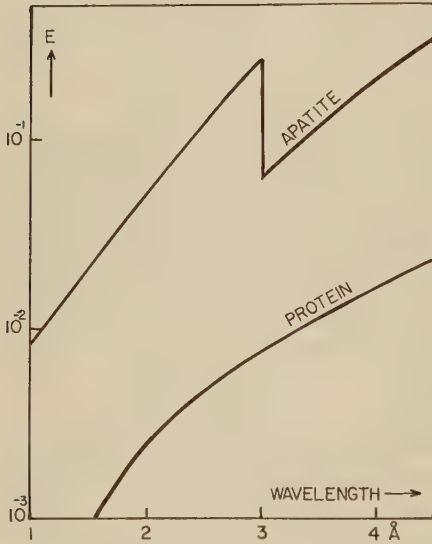


Fig. 1.

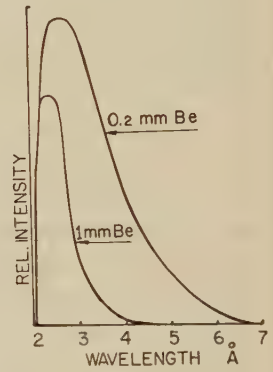


Fig. 2.

Fig. 1. Extinction of X-rays of various wave lengths by sections of apatite and protein one micron thick.

Fig. 2. Distribution of wave lengths of X-rays in the microradiographic experiments.

sorption coefficient and ρ the density; m is the mass in gm cm^{-2} of the absorbing substance; indices i and o indicate the inorganic and organic material respectively. Only a small fraction of the X-ray attenuation is due to scattering and it can be neglected.

The extinction $\left(\ln \frac{I_o}{I}\right)$ at various wave lengths has been calculated for 1μ thick layers of apatite, a major inorganic constituent, and organic material (Fig. 1). It is seen that the X-ray extinction of the apatite is 10 to 20 times higher than that of the organic material of the same thickness. The volumes occupied by the organic and inorganic components in bone are roughly equal. Therefore, in a section of bone, with the wave lengths indicated in the diagram (Fig. 2), more than 90 per cent of the absorption can be attrib-

uted to the inorganic phase. A microradiogram registered at these wave lengths therefore mainly shows the distribution of mineral salts.

Interference microscopy. It has been shown (5) that the mass of material other than water (the dry mass) per unit area, m , in a homogeneous object of thickness t situated in water is given by

$$m = \frac{\Phi_w}{\chi} \dots \dots \dots 2)$$

where Φ_w is the o.p.d. in water; that is $(\mu_o - \mu_w)t$ where μ_o and μ_w are the refractive indices of the object and water respectively. For solutions or gels χ is 100α , where α the specific refractive increment is given by $(\mu_o - \mu_w)/c$ and c is the concentration in gm/100 cc. For a solid dry object of refractive index μ_o , χ is $(\mu_o - \mu_w)/\rho$ where ρ is the density of the object. For the major substances (nucleic acids, proteins, lipoproteins) occurring in soft tissues the values of χ are approximately the same with an average value ~ 0.18 . Hence by measuring Φ_w the dry mass per unit area can be determined.

Equation 2) cannot, however, be applied directly to bone tissue which contains organic material which may be at a high concentration and also mineral salts in a crystalline form. While χ for a solution of protein in concentrations up to 0.1 gm/cc is 0.19, χ for a dry protein is ~ 0.15 . It will be assumed that the main mass of the extracellular organic matrix of bone is collagenous protein. Since the concentration of the protein is not known the average value 0.17 will be used for χ -organic (χ_o). The chief inorganic constituent of bone is a hydroxyapatite and the value 0.1 for χ_i has been calculated from the refractive index (1.64) and density (3.13) by using an expression given above and neglecting the small fraction of water. A small fraction is calcite which is doubly refracting with χ -values 0.06 and 0.13. The value 0.1 will be used for χ -inorganic (χ_i). The appropriate average value of χ for bone is then

$$\chi_{av} = \chi_i \frac{m_i}{m} + \chi_o \frac{m_o}{m} \dots \dots \dots 3)$$

where m is the sum of m_i and m_o which are the masses of inorganic and organic substances per unit area respectively. With this value for χ equation 2) gives

$$\Phi_w = \chi_i m_i + \chi_o m_o \dots \dots \dots 4)$$

Since in intact bone the ratio m_i/m_o is very approximately 3 and χ_i/χ_o is 0.6, about 65 per cent of the observed o.p.d. in water is due to the inorganic substance. By measuring Φ_w , m_i can be determined if m_o is known.

When a fixed section of intact bone of geometrical thickness t is placed in a fluid medium it may be assumed that the medium will permeate any spaces which may exist between the material particles comprising the bone section. Let μ_o be the refractive index of the bone particles and let the medium permeate a fraction f of the volume of the section. Then neglecting 'distribution error' (see discussion) the o.p.d. of the section in a medium of refractive index μ_m is given by

$$\Phi_m = (\mu_o - \mu_m) t (1 - f) \dots \dots \dots 5)$$

By measuring the o.p.d.s (Φ_m , Φ_w) of the section in two liquids (7, 4) of different refractive indices (μ_m , μ_w), the 'effective thickness' of the section obtained from two equations of the form 5) is

$$t(1 - f) = \frac{\Phi_w - \Phi_m}{\mu_m - \mu_w} \dots \dots \dots 6)$$

Hence the localised concentration or density ρ (gm/cc) of the bone material is

$$\rho = \frac{(m_o + m_i)(\mu_m - \mu_w)}{(\Phi_w - \Phi_m)} \dots \dots \dots 7)$$

EXPERIMENTAL

Thin slices of bone from human autopsy material were cut by a small rotating saw, and then ground under absolute ethanol on glass plates to a final thickness of about 5 to 10 μ . Small fragments of such thin sections of bone were required for interference microscopy.

The bone sections were microradiographed with 6 kV radiation from either a Machlett OEG 50 tube (inherent filtration 0.2 mm Be + 50 mm air) or a Machlett AEG 50 T X-ray tube (1 mm Be window). Fig. 2 shows the intensity distribution in the X-rays generated in these tubes. The microradiograms were recorded on Eastman Kodak Spectroscopic Plate 649 and then optically enlarged by photomicrography. The X-ray absorption of chosen structures was determined by photometry of the microradiogram, and the X-ray absorption of the bone structures were expressed in units of a reference system which was microradiographed simultaneously.

After microradiography the bone sections were transferred and mounted in either liquid paraffin or water for observation and measurement in a Cooke, Troughton and Simms, Dyson interference microscope, with an objective of numerical aperture 0.85 and a condenser of numerical aperture ~ 0.5 . The microscope was used either with interference bands in the field or with the field evenly illuminated—that is, with infinite separation of the interference bands.

Measurements of o.p.d. at chosen points in the bone section were made with the microscope adjusted to give interference bands. The section displaced the interference bands and the o.p.d. could be determined by measuring this band displace-

ment as follows. The sections were first examined in white light illumination when the interference bands showed the colour sequence of Newton's rings with a unique white central band along which the optical path difference was zero. The number of bands between this unique white band in the section and the corresponding white band in the clear field was noted approximately. In monochromatic light of wave length λ *in vacuo*, if D is the distance between the minima of corresponding bands in clear field and section, then the o.p.d. Φ at the point in the section located on that band minimum is D/d wave lengths where d is the distance between the bands in the clear field. D and d were measured from suitably enlarged photomicrographs taken with monochromatic green light, λ 546 m μ , isolated from a B.T.H. 250 watt compact mercury arc by Wratten filters 77A and 58. Photomicrographs were taken on either Kodak Microfile Pan or Ilford Pan F 35 mm films. Since the bone section sometimes obscured part of the interference bands in the clear field, their position and direction were marked on the photomicrograph by a cross-hair located in the primary image plane in the projection eyepiece.

The o.p.d.s were measured in this way at the chosen points in a bone section mounted first in liquid paraffin, then in water, and finally, after decalcification, in 0.1 per cent hydrochloric acid.

Photomicrographs of bone sections were also recorded when the microscope was adjusted so as to give infinite separation of the interference bands. Under these conditions, provided that the variations in o.p.d. in the section were in the range $r\lambda$ to $(n + \frac{1}{2})\lambda$ ($n = 0, 1, 2, 3$ etc.) the light intensity in the image increased with the o.p.d. and hence mass per unit area. Similarly lighter regions in the X-ray microradiogram were those with higher mass per unit area.

Decalcified sections were also photographed in polarised light.

RESULTS

The X-ray microradiogram (Fig. 3) of a cross-section of an adult human femur (compacta) shows varying amounts of mineral salts in the different Haversian systems. In some systems there is a gradient in the mineralisation with the largest amount close to the Haversian canal. The lacunae of the osteocytes appear as small black dots. They are not mineralised and hence do not absorb the X-rays.

A thin section of bone was photographed first in X-ray contrast and then in dark-field interference contrast with infinite separation of the interference bands (Fig. 4 a, b). The variations in o.p.d. in the mineralised regions were found, by preliminary measurements in white light with interference bands, to range from 1 to 1.5 λ . The distribution of light intensity, and hence that of dry mass per unit area, as shown by X-rays and optical interference is seen to be strikingly similar in the mineralised regions. However, the interference picture also shows the organic material which occurs in annular regions inside the Haversian Canals. Some of the organic material inside

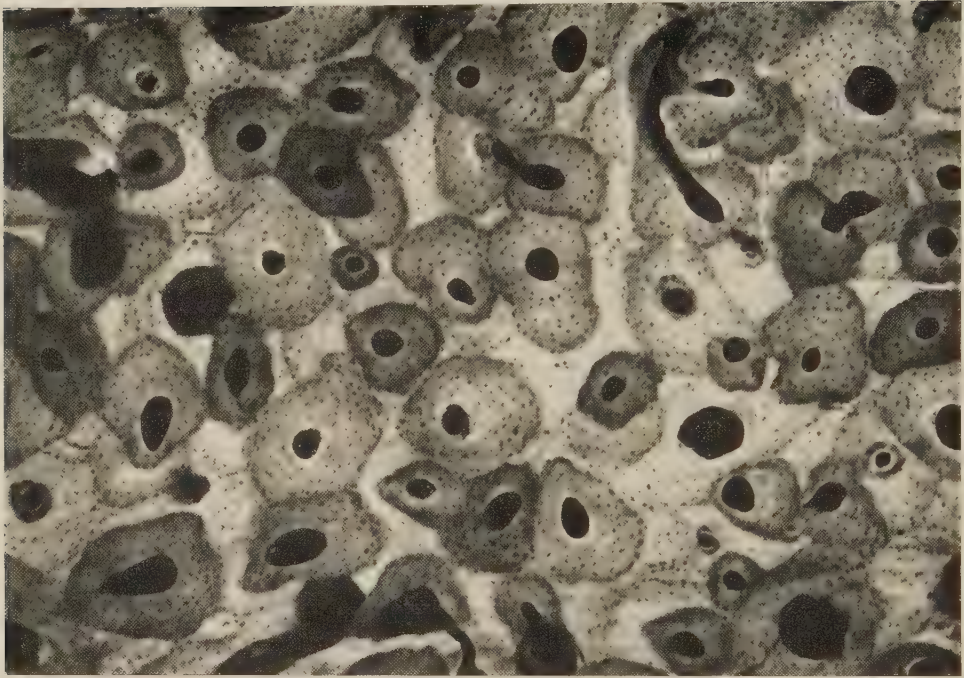


Fig. 3. Enlarged microradiogram of a cross section of bone. The varying degree of mineralization is clearly shown. The osteocyte spaces are free of inorganic material. Practically all X-ray absorption is due to the inorganic material. Mag. $\times 80$.

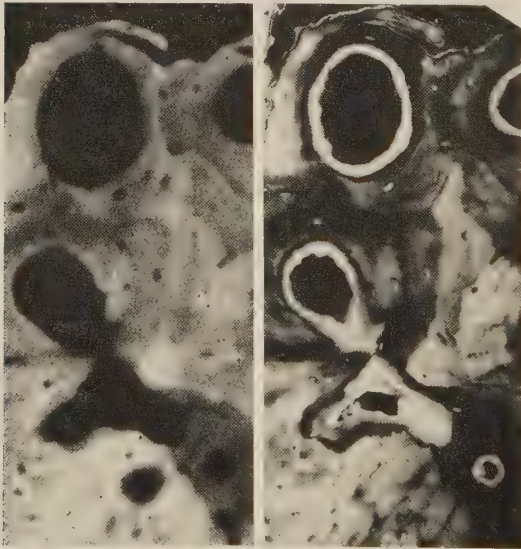


Fig. 4. To the left a) a microradiogram of a thin section of bone showing varying degrees of mineralization, and to the right b) an interference contrast image of the same sample cf. the text. Mag. $\times 140$.

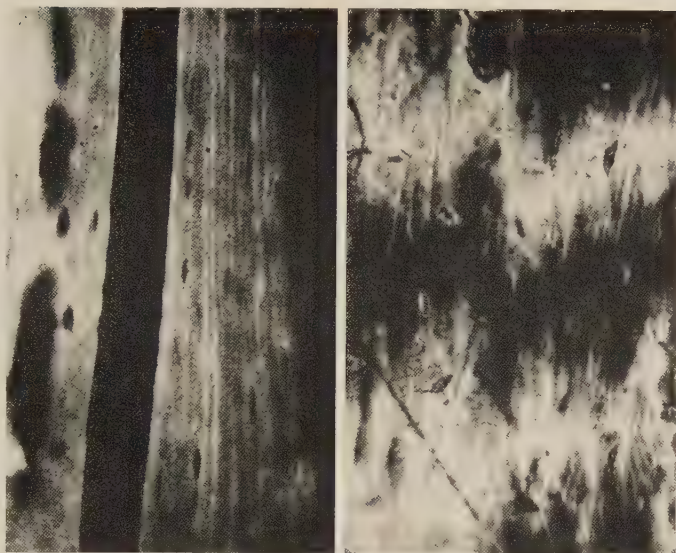


Fig. 5. a) Microradiogram at high magnification of a longitudinal section of a Haversian system showing the layered mineralization. b) The interference contrast image which also demonstrates the layered structure. Mag. $\times 240$.

the Haversian canals may have been deposited during the grinding process. The o.p.d. of these regions is about 0.5λ and hence they appear light in the photomicrograph. The o.p.d.s in the lacunae of the osteocytes range from $0-0.5 \lambda$ and hence they appear as either light or dark regions.

The X-ray microradiogram (Fig. 5 a) of a longitudinal section of a Haversian system shows the varying contents of mineral salts in the lamellae. In the photomicrograph (Fig. 5 b) of a slightly different region of the same section, taken with infinite separation of the interference bands, the few broad bands show that the section is wedge-shaped. The varying contents of the lamellae show up as narrow light and dark lines at right-angles to the bands.

In the X-ray microradiogram (Fig. 6 a) of a transverse section of bone tissue the Haversian system appears darker and hence has a lower mass of mineral salts per unit area (and also concentration) than the old bone tissue surrounding it. The same region was photographed (Fig. 7 a) in the interference microscope adjusted to give interference bands. The band displacement in the old bone tissue is greater than it is in the Haversian system (distance $l_1 > l_2$ in Fig. 7 a). Hence the mass per unit area is greater in the former region than in the latter in agreement with the X-ray data. The cana-

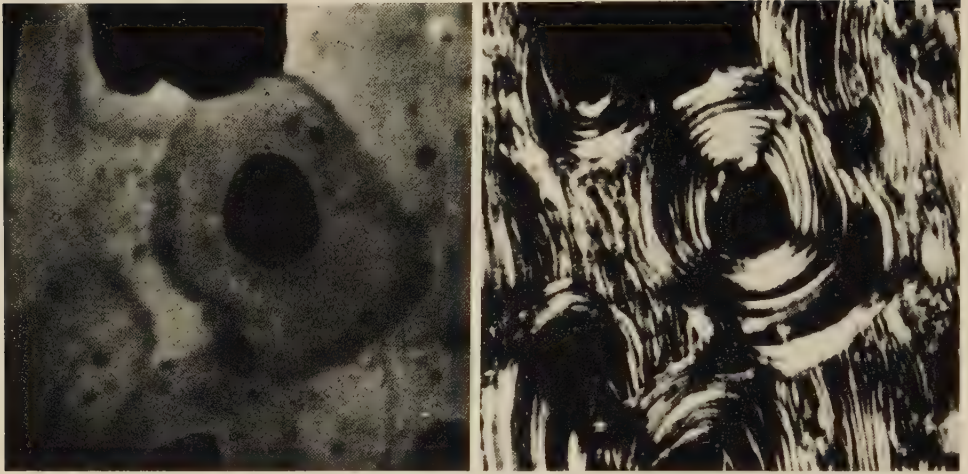


Fig. 6. a) Microradiogram of a thin cross section of a few Haversian systems. Note low mineral content of the system. b) The same area after decalcification photographed in polarized light. Mag. $\times 160$.

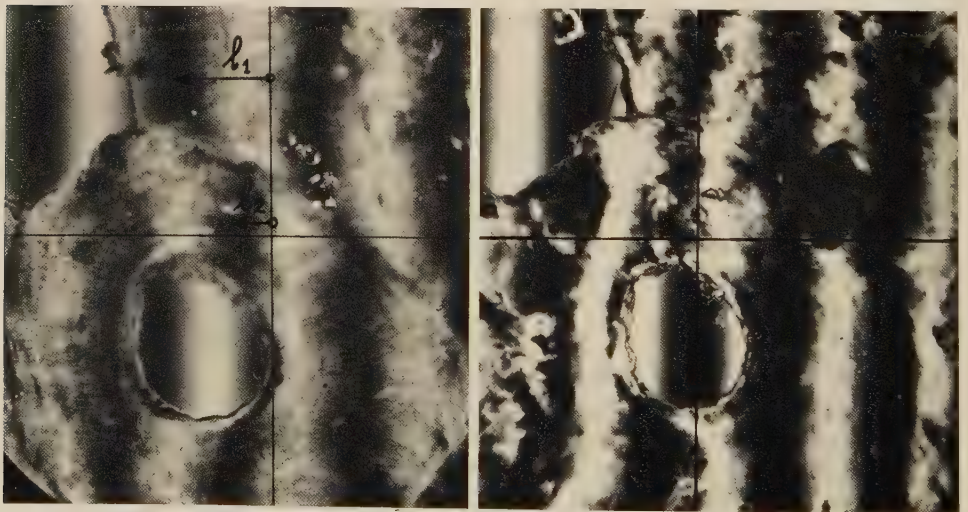


Fig. 7 a) The same sample as in Fig. 6 a) showing how the interference bands are shifted. The band shift indicates less material (mainly inorganic) in the Haversian system than in the surrounding bone tissue $l_2 < l_1$. b) The same section after decalcification. Note that the bands go straight through the specimen indicating a uniform distribution of organic material. Mag. $\times 240$.

liculae show up as a network of fine lines in Fig. 7 a. In some regions of the Haversian system the circularly arranged lamella structure may be seen as alternate light and dark fluctuations superimposed on the interference bands.

After decalcification (Fig. 7 b) there is no discontinuity between the interference bands in old bone and Haversian system and hence the dry mass of organic material per unit area is approximately the same in both regions. In certain regions of Haversian system in the decalcified section it was sometimes possible to distinguish alternate light and dark fluctuations superimposed on the interference bands, indicating circularly arranged lamellae. Fig. 6 b shows the typical appearance with polarised light of the collagen fibrillae in the decalcified section.

Quantitative measurements were made on one bone section containing four concentrically laid down Haversian systems (A-D). The Haversian systems were termed young (y) while the irregular regions neighbouring them were termed old (o). These latter regions were fragments of old Haversian systems. The o.p.d.s. in wave lengths and X-ray extinctions were measured at several points (numbered in the following tables) in any one Haversian system and surrounding old bone tissue. The dry mass of organic substance per unit area (m_o in Table I, Col. 5) was first determined from the o.p.d.s of the decalcified section in water by using equation 4) with $m_i = 0$. The dry mass of inorganic substance per unit area (m_i) was next calculated from the o.p.d. measurements in water before and after decalcification. The ratio of inorganic to organic content (Table I, col. 8) is lower in the Haversian system than in the surrounding old bone tissue. The X-ray extinctions measured at the same points in the section are proportional to a close approximation (see theoretical discussion) to the mass of inorganic substance per unit area. The variations in the values of X-ray extinction throughout the section are due to differences in both the concentration of inorganic substance and the thickness of the section. However, the ratio of these values to those for the inorganic content determined by interferometry are approximately constant (Table I, col. 10).

The ratios of inorganic material at adjacent points in old (o) and young (y) bone tissue were measured by X-rays and interferometry. The two methods gave approximately the same results (Table II).

From the measurements of o.p.d. at identical points in the bone section mounted first in liquid paraffin ($\mu = 1.482$) and then in water ($\mu = 1.333$) the effective thickness ($t(1-f)$ in Table III, col. 5) was calculated from equation 6. The effective thickness in old bone (o) was found to be greater than

TABLE I

Haver- sian system	Posi- tion	o.p.d. in water before decalc. λ	o.p.d. in water after decalc. λ	m_o gm/cm ² ($\kappa = 0.17$)	Difference in o.p.d. before and after decalc. λ	m_i gm/cm ²	m_i/m_o	X-ray extinc- tion arb. units	X-ray extinction o.p.d. difference
A	1 Y	2.70	1.20	3.9×10^{-4}	1.50	8.2×10^{-4}	2	23	15
	2 O	3.40	1.30	4.2	2.10	11.5	2.7	29	14
	3 O	3.00	1.15	3.7	1.85	10.1	2.7	28	15
	4 Y	2.35	1.15	3.7	1.20	6.5	1.8	20	17
	5 Y	2.75	1.25	4.0	1.50	8.2	2.0	23	15
	6 Y	2.35	1.20	3.9	1.15	6.3	1.6	23	20
	7 Y	2.25	1.25	4.0	1.50	8.2	2.0	25	17
	8 O	3.05	1.35	4.3	1.70	9.3	2.2	27	16
	9 O	2.70	1.15	3.7	1.55	8.5	2.3	26	17
	10 Y	2.70	1.15	3.7	1.25	6.8	1.8	24	19
	11 Y	2.70	1.35	4.3	1.35	7.4	1.7	24	18
	12 O	2.60	0.90	2.7	1.70	6.3	3.4	25	15
B	1 O	4.40	1.80	5.8×10^{-4}	2.6	14.2×10^{-4}	2.4	39	15
	2 Y	3.50	1.80	5.8	1.7	9.3	1.6	29	17
	3 O	4.75	1.80	5.8	2.15	16.1	2.8	41	14
	4 Y	3.85	1.80	5.8	2.05	11.2	1.9	34	16
C	1 O	3.25	1.30	9.2×10^{-4}	1.95	10.7×10^{-4}	2.5	33	17
	2 O	3.75	1.60	5.1	2.15	11.8	2.3	30	14
	3 O	3.70	1.40	4.5	2.30	12.6	2.8	32	14
	4 O	3.60	1.30	4.2	2.20	12.6	3.0	30	13
D	1 Y	4.15	2.0	6.4×10^{-4}	2.15	11.8×10^{-4}	1.8	33	15
	2 Y	4.40	2.0	6.4	2.40	13.1	2.0	35	15
	3 O	4.80	2.0	6.4	2.80	15.4	2.4	37	13
	4 O	5.0	2.0	6.4	3.00	16.4	2.6	41	14

TABLE II

Ratio of mineral salts at adjacent points in O and Y bone tissue	Int.	X-ray
(A) 3 to average of 1 and 5	1.23	1.22
(A) 8 to average of 10 and 11	1.31	1.13
(A) Average of 8 and 9 to 10	1.31	1.10
(B) 1 to 2	1.53	1.35
(D) 3 to average of 2 and 1	1.22	1.09
(D) 4 to 2	1.25	1.17

TABLE III

Haversian system	Position	o.p.d. in paraffin. Intact bone λ	Difference between o.p.d. of intact bone in water and paraffin λ	Effective thickness: $t(1-f)$ μ	$m_i + m_o$ gm/cm ²	Density gm/cc
A	1 Y	0.86	1.85	6.5	12.1	1.8
	2 O	1.25	2.25	8.0	15.7	1.9
	av. 5, 4 Y	0.85	1.70	6.5	11.0	1.75
	av. 6, 7 Y	0.80	1.70	6.5	11.0	1.8
	8 O	1.15	1.90	7.0	13.6	2.0
	10 Y	0.75	1.60	6.0	10.5	1.8
B	2 Y	1.20	2.95	8.5	15.8	1.8
	3 O	1.75	2.30	11.0	21.9	2.0
C	1 O	1.30	1.95	7.0	14.9	2.1
	av. 2, 3 O	1.40	2.30	8.5	17.0	2.0
D	1 Y	1.60	2.55	9.5	18.2	1.95
	2 Y	1.10	2.80	10.5	19.5	1.9
	3 O	1.80	3.0	11.0	21.5	2.0
	4 O	1.85	3.15	11.5	22.8	2.0

at an immediately adjacent point in young (y) bone where the geometrical thickness t was the same. This was due to the greater amount of substance in the old bone which reduces the value of f , the fraction of the thickness occupied by the immersion medium. The density of the bone was calculated from equation 7), (see Table III, col. 7).

DISCUSSION

The varying degree of mineralisation in adult bone tissue has been clearly demonstrated by interferometry and agrees with what has earlier been shown by X-ray microradiography (1, 2). The age of the Haversian systems formed in the resorption cavities in the compact bone is difficult to assess. Hence in the tables of results the term young (y) applied to the concentrically laid down Haversian systems includes systems of varying age. Interferometric studies of decalcified bone have shown little variation in the organic content of old (o) and young (y) regions of bone.

The accuracy of the data obtained by interferometry depends upon several factors. In the measurement of o.p.d. of any inhomogeneous object there

may be a 'distributional' error; that is, the o.p.d. may not be the same as if the material were distributed homogeneously throughout the same volume. The contrast in the interference bands in the image of the object may be used as a rough measure of the inhomogeneity. When the contrast is high, as in the bone sections, the errors will be small. The maximum possible error in the values for the dry mass of organic material due to the uncertainty in the value of χ may be deduced from the data given above. It is about ± 12 per cent. No differences could be detected in the area of the intact bone in liquid paraffin and in water, and of the decalcified bone in water. Hence it is permissible to use the values obtained for m_o , the mass per unit area of organic material in the decalcified tissue, in the calculation of m_i , the inorganic mass per unit area, in equation 4). It can also be shown that any change in the refractive index of the intact bone tissue when in water does not affect the values for the effective thickness or the density, which refer to the bone tissue as it exists in liquid paraffin. There is the possibility that during the decalcification of the bone there may have been a redistribution and loss of organic material. The orientation of the collagen fibrillae in the decalcified section as shown by polarised light indicates that there is no major change in the distribution of this material. Substances other than collagen and protein (e.g. mucopolysaccharides) occur in relatively small amounts and possible losses could have little effect on the quantitative results. No measurements were attempted on the amounts of material in the lamellar structure showing up in the images in interference contrast as small fluctuations of light intensity superimposed on the interference bands. The nature of these fluctuations might also depend on the birefringence of these regions.

The quantitative data show that there is good agreement between the relative amounts of inorganic material obtained by the two methods (Table I, col. 10 and Table II). The effect of the small absorption of organic material by the X-rays (see theoretical) is to increase the ratios in column 3 of Table II by 5–10 per cent. The average density of intact bone tissue obtained by the interferometric method is 1.9 and this is in good agreement with the value 2.0 to 2.1 obtained by the conventional method on macro-samples of bone.

SUMMARY

Microinterferometric and microradiographic methods have been applied to the study of the composition of bone tissue. The interferometric technique (with the Dyson interference microscope) confirmed the varying degree of

mineralization demonstrated by soft X-ray micrography. There was a good agreement between the relative amounts of inorganic material in various structures as revealed by X-rays and interference contrast. Interferometry before and after decalcification gave the ratio inorganic to organic material and there was a uniform distribution of the organic material.

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THE EFFECT OF NON-IONIC DETERGENTS ON THE GROWTH OF *TETRAHYMENA*¹

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THE stimulatory effect of certain detergents on the growth of *Tetrahymena* was first reported in 1949 (6). The use of the various Tweens was suggested by the earlier work of Dubos (4), Davis and Dubos (2) and Williams, Broquist and Snell (10) who showed the stimulatory effects of fatty acids on certain bacteria, especially when the fatty acids were present in combined form. It was early noted that, in *Tetrahymena*, not all detergents caused growth stimulation but only those containing oleic acid (Tween 85 and Myrj G2144). At the concentration first used, Tween 80 (a monooleate) was without effect, but later studies (5) indicated that Tween 80 was to be preferred to Tween 85 (a trioleate) when high concentrations of the former were employed. The main advantage of the monooleate was the clear medium resulting, making turbidimetric measurements of cell concentration much more accurate. Since these observations were made, Tween 80 has been added routinely to the medium for *Tetrahymena pyriformis* W.

Difficulty was encountered when certain types of work on *Tetrahymena* were initiated, for it was found that organisms grown in the presence of Tween 80 were far from ideal for handling when chemical extractions of the cells were the object. The cells tend to form a layer at the surface of the fluid during centrifugation and, in addition, are very fragile. This makes washing procedures and concentration of organisms almost impossible. These observations led to further study of the cells grown in the presence and absence of Tween and other detergents. We have found that the stimulation by the Tweens actually only occurs during the relatively early growth phases and is of a smaller magnitude than originally supposed. The increased yield, as judged by turbidimetric measurements, was found to be due not to increased numbers of cells per unit volume of culture medium but to larger and more opaque cells.

¹ This investigation was supported in part by a grant from the United States Atomic Energy Commission (Contract No. AT(30-1)-1351) and a grant from the National Institutes of Health.

MATERIAL AND METHODS

Tetrahymena pyriformis (geleii) W (1) was grown as previously described (3, 7). The basal medium is given in Table I. To this medium were added the various fatty acid derivatives and other non-ionic detergents. Turbidimetric measurements were made on a Lumetron colorimeter (Model 401) using a 650 filter. Cell counts were made by the direct method described elsewhere (5). Nitrogen determinations were made by standard micro-Kjeldahl procedures. Lipid determinations were made by extracting the washed organisms repeatedly with hot ethanol-ether mixtures and weighing both the extracted material and the cell residue after drying.

For demonstration of lipid material within the cells, the organisms were dried rapidly on glass slides, dehydrated to 60 per cent ethanol, stained with Sudan III in ethanol and mounted in glycerine. The organisms so stained were then photographed both with standard film and in color.

TABLE I
Composition of basal medium.

	γ/ml		γ/ml
DL-alanine	110	Guanylic acid	30
L-arginine	206	Adenylic acid	20
L-aspartic acid	122	Cytidylic acid	25
Glycine	10	Uracil	10
L-glutamic acid	233		
L-histidine	87	Dextrose ¹	2,500
DL-isoleucine	276	Na acetate	1,000
L-leucine	344		
L-lysine	272	MgSO ₄ · 7H ₂ O	100
DL-methionine	248	Fe(NH ₄) ₂ (SO ₄) ₂ · 6H ₂ O	25
L-phenylalanine	160	MnCl ₂ · 4H ₂ O	0.50
L-proline	250	ZnCl ₂	0.05
DL-serine	394	CaCl ₂ · 2H ₂ O	50
DL-threonine	326	CuCl ₂ · 2H ₂ O	5
L-tryptophane	72	FeCl ₃ · 6H ₂ O	1.25
DL-valine	162	K ₂ HPO ₄	1,000
		KH ₂ PO ₄	1,000
Ca pantothenate	0.10		
Nicotinamide	0.10		
Pyridoxal HCl	0.10		
Pyridoxamine HCl	0.10		
Riboflavin	0.10		
Folic acid	0.01		
Thiamine HCl	1.00		
Biotin (free acid)	0.0005		
Choline Cl	1.00		
6-Thioctic acid	0.004		

¹ Autoclaved separately and added aseptically.

RESULTS

The various detergents investigated fall roughly into three categories as judged by their effect on the growth of *Tetrahymena* (Table II). The oleic acid esters of alkylene oxide hexitan or of alkylene glycol were stimulatory, as judged by turbidimetric measurements. Myrj G2144, which is the monooleate of polyoxyethylene oxide (without the hexitan) produced stimulation only after a period of depression, so while four day readings equalled those obtained by the addition of Tween 80 earlier readings were lower than the controls. One group of detergents proved to have no effect on the turbidity readings, while the third group was definitely inhibitory. These results were obtained after tests through wide concentration ranges.

The stimulatory effect could conceivably be due to any one of a number of mechanisms: a) surface activity effects; b) detoxification, especially of fatty acids; c) a source of fatty acid used by the organisms for synthesis and/or energy. Each mechanism may contribute to the effect but no single one seems to be the whole answer.

The surface activities of the various Tweens are reported (Atlas Surface Active Agents, Atlas Powder Company, Wilmington, Delaware) to be very nearly identical for a given concentration of material, although they differ decidedly in their growth effects on *Tetrahymena*. Biotin also has been reported (11) to be surface active but this compound has no effect on the growth of *Tetrahymena* over wide concentration ranges.

TABLE II

Comparison of growth effects of various detergents as judged by turbidimetric measurements after 96 hours of growth.

Stimulatory	Inert	Inhibitory
Tween 80 ¹	G2320 ¹	Triton X100 ⁴
Tween 85 ¹	Tween 60 ¹	Span 60 ¹
Myrj G2144 ^{1, 2}	Triton WC 1339 ⁴	Span 80 ¹
Polyethylene glycol monooleate ³	Ethomid HT/60 ⁵	Ethomid C/25 ⁵
	Ethomid RO/25 ⁵	Polyethylene 40 monoricinoleate ³

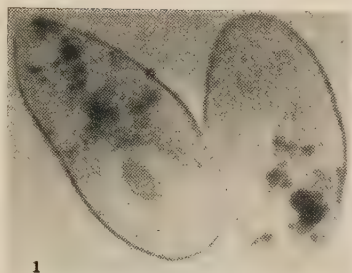
¹ Atlas Powder Co. (see Table III).

² Myrj G2144 stimulates maximum yield (turbidimetric readings) but is actually inhibitory during earlier growth phases.

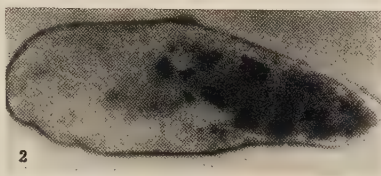
³ Glyco Products Co., Inc.

⁴ Rohm and Haas Co. (alkyl aryl polyether alcohol).

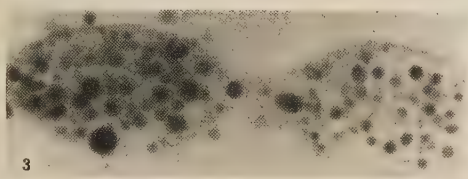
⁵ Armour Chemical Division (polyoxyethylene N-substituted fatty acid (whole oil) amides).



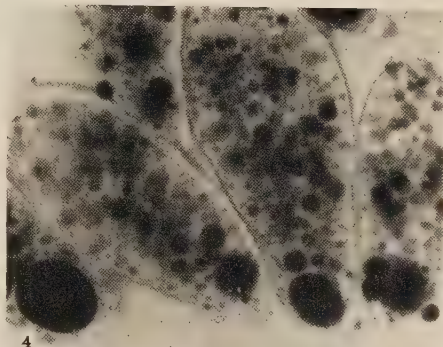
1 CONTROL 4 DAYS



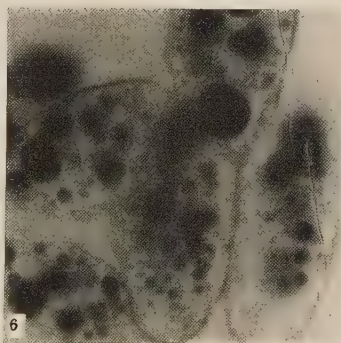
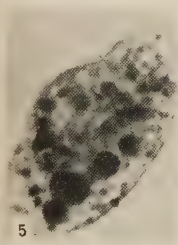
2 TWEEN 60 4 DAYS



3 TWEEN 85 8 DAYS



4 TWEEN 85 4 DAYS



6 TWEEN 80 3 DAYS



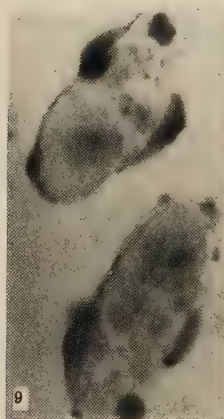
7 TWEEN 80 4 DAYS

It has previously been shown in bacteria (10) that the Tweens are effective in overcoming the toxicity of free fatty acids. This is true to a certain degree



8

SPAN 80 2 DAYS



9

SPAN 80

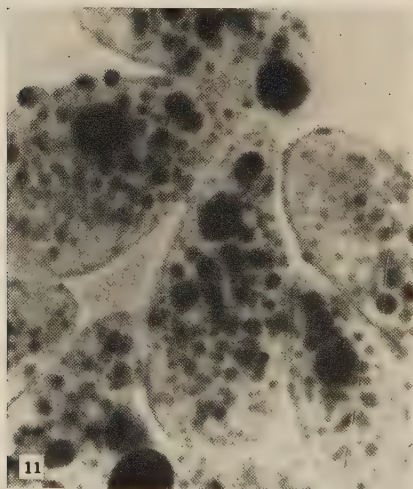
3 DAYS



10

SPAN 80

4 DAYS



11

MYRJ G 2144

4 DAYS



12

G 2320 4 DAYS

for *Tetrahymena*. Long chain free fatty acids are inhibitory but become much less so when Tween 80 or 85 is included in the medium.

Experiments were conducted to determine the effect of certain of the detergents on cell number during the development of the population, as opposed to turbidity of the cultures. Tween 80, Tween 85, Myrj G2144 and

G2320 (Table III) were chosen. All sets, including detergent-free controls, were inoculated from the same cell suspension with an automatic pipetting machine to insure equality of numbers and physiological state of the organisms used (7). As seen in Table IV all cultures produced nearly equal numbers of cells after four days incubation, although the growth rate during the earlier periods was greater than the controls for the Tweens, less for Myrj and nearly equal for G2320. Turbidimetric readings, however, were widely divergent.

Microscopic examination of the cells revealed that the Tween and Myrj-grown cells were distended with lipid droplets and were quite opaque, while the control cells and those grown in the presence of G2320 were smaller and quite clear.

Extensive examination of Sudan III stained cells revealed that whenever fatty acid esters were present, whether or not they produced stimulation of growth, the organisms appeared to break down the detergent complex, storing

TABLE III

Comparative structure of certain of the surface-active agents used.¹

Name	Alkylene Oxide	Hexitol or Hexitan	Fatty Acid
Tween . . .	polyoxyethylene	sorbitan	80-monooleate 85-trioleate
Span	—	sorbitan	80-monooleate
Myrj	polyoxyethylene	—	G2144-monooleate
G2320 . . .	polyoxyethylene	sorbitol	—

¹ From Atlas Surface Active Agents, Atlas Powder Co., Wilmington, Del.

TABLE IV

Comparison of cell number and optical density.

	Number cells per ml				Optical density × 1000			
	43 hrs.	54 hrs.	67 hrs.	98 hrs.	43 hrs.	54 hrs.	67 hrs.	98 hrs.
Control (no detergent) .	147,000	215,000	300,000	520,000	102	187	237	285
Tween 80	210,000	380,000	440,000	560,000	240	444	600	710
Tween 85	210,000	394,000	460,000	530,000	352 ¹	475	629	678
Myrj G2144	14,400	48,000	164,000	460,000	41	74	194	577
G2320	180,000	270,000	330,000	500,000	143	222	256	288

¹ Turbid medium.

the fatty acid in droplets and pools within the cytoplasm. As the cultures aged some of the larger of these droplets were extruded into the medium, adding to its turbidity.

A comparison of the Sudan III stained organisms is shown in the photomicrographs. Fig. 1 shows the control organisms after four days of growth. Lipid droplets are present, as would be expected from earlier observations (10, 11), but to only a limited extent. Fig. 2 represents the storage of lipid from a stearic Tween. The use of Tween 85 and Tween 80 results in enormous lipid storage (Figs. 3-7). The actual lipid extrusion is shown in Figs. 8-10 where Span 80 (inhibitory) is used. Extensive storage of lipid results when Myrj G2144 is used (Fig. 11). These cells do not appear different from those grown in the presence of Tween 80 or Tween 85. When no fatty acid is present, as in G2320, the cells appear much like the controls (Fig. 12). Some lipid material is synthesized and stored. One feature of the G2320-grown cells, which is not apparent in the photographs, is the somewhat heavier pellicle, making them more rigid than the controls.

TABLE V

Comparison of lipid content of cells grown in presence and absence of Tween (96 hr. cultures).

	Lipid-free cells (mg dry wt.)	Lipid (mg dry wt.)	Per cent lipid
Control . . .	40.9	8.7	17.5
+ Tween ¹ . .	38.6	31.3	44.8

¹ Tween 80 at 10 mg/ml.

TABLE VI

Comparison of nitrogen content of cells grown in the presence and absence of Tween.

Age of culture in hrs.	Type of culture	Optical density × 1000	Mg cellular N per ml culture
48	Control	76	0.054
	+ Tween 80 ¹	131	0.039
72	Control	284	0.100
	+ Tween 80 ¹	523	0.103
96	Control	325	0.114
	+ Tween 80 ¹	685	0.127

¹ Tween 80 at 10 mg/ml.

Lipid determinations of washed cells show that the controls contain over 17 per cent lipid material, which is in good agreement with the figure given by McKee *et al.* (8) for peptone-grown *Tetrahymena*, while the Tween-grown organisms contained over 44 per cent lipid material (Table V).

Nitrogen determinations (Table VI) revealed no significant difference between the total cellular nitrogen of the control and Tween 80-grown cultures on a unit volume basis. It would appear from these results, however, that each cell contains more protein and/or free amino acids in the early than in the later growth phases, and that the control cells are somewhat higher in nitrogen content than the Tween-grown cells (compare cell numbers at the three time intervals in Table IV). We have no explanation for this difference.

DISCUSSION

It seems possible that the oleic acid can act as a readily utilizable source of energy if it is presented in the correct complex; omitting the alkylene oxide, as in Span 80, results in an inhibitory compound. With the present evidence at hand, therefore, it is impossible to assign stimulatory function to any one of the specific parts of the Tween molecule, except the oleic acid.

When four-day growth is taken as the end point and measurements are made turbidimetrically, the use of Tween 80 is desirable, for it allows one to use more of the colorimeter scale and small differences of response are easily detectable. When organisms are to be grown for enzymatic experiments, extractions and fractionation purposes, however, the high lipid content of the cells together with their fragility make its use impractical.

SUMMARY

An investigation of the effects of a number of non-ionic detergents on the growth rate, maximum yield, and cytology of *Tetrahymena pyriformis* W was carried out. It was found that certain oleic acid-containing detergents were stimulatory during early growth phases. Other detergents were either inhibitory or inert. It was found that the organisms appeared to break down the detergent molecule and to store large quantities of the fatty acid. The cell numbers at the end of a four-day growth period were the same for Tween-grown organisms and the controls, as was the cellular nitrogen content. The turbidimetric readings, however, were very much lower for the controls. The differences in turbidity were found to be due to the larger lipid-filled

and more opaque cells in the Tween cultures. The use of detergents is not recommended when *Tetrahymena* is to be used for experiments where cells must undergo manipulative procedures.

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THE STRUCTURE OF CHLOROPLASTS

IV. THE DEVELOPMENT AND STRUCTURE OF THE *ASPIDISTRA* CHLOROPLAST

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The investigation herein described is a continuation of former studies in chloroplast structure (3, 4), concurrent with the publication of which there have also appeared other reports on the same subject (1, 2, 7, 8, 9, 10).

MATERIAL

Potted specimens of *Aspidistra elatior* were used and leaves in different stages of growth were selected during the months of March to May as the basis for observation of chloroplast development. The plants were placed near a window exposed to unimpeded day-light.

METHODS

Samples of the leaf were excised by means of a glass-knife and were fixed with osmium tetroxide at room temperature and in darkness for 3-4 hours, the technique consisting of suspending the samples by threads in the vapor of the fixing compound derived from a 2 per cent solution held in a closed vessel.

Reference may be made to the method for dehydration, embedding, and sectioning described in a previous paper (5). A Siemens electron microscope Model 1939 was used with a primary magnification of $8500\times$ at a potential of 90 kv.

RESULTS

Early stages in the development of the chloroplast. Young shoots of *Aspidistra* are constructed of two outer protecting leaves which enclose the leaf proper. The cells of this inner leaf show a sparse structure as would be expected from their high water content. The protoplast is concentrated near the cell walls and here appear dense bodies (Fig. 1) which are also to be found, sometimes quite numerous, together with characteristic chloroplasts in

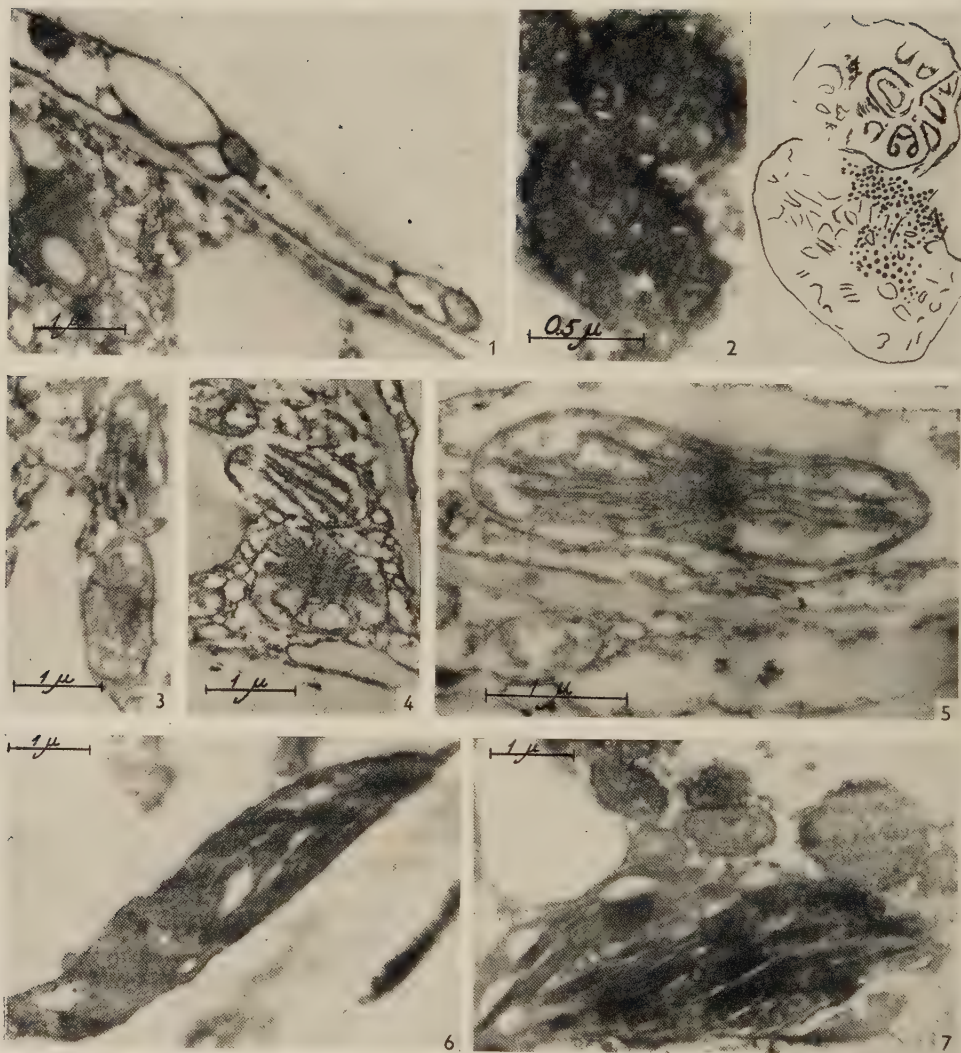


Fig. 1. Dense bodies in the protoplast near the cell wall forming very early stages of chloroplast development.

Fig. 2. The inner structure of some dense bodies. (The connection between these bodies and chloroplasts has not been conclusively established so far.)

Figs. 3-4. The first sign of lamina-formation in chloroplasts of *Aspidistra*.

Fig. 5. Young plastid with a few extended double-laminae.

Fig. 6. Young plastid with a few laminae.

Fig. 7. Young plastid with groupings of laminae and "dense bodies" outside the chloroplast.



Fig. 8. Chloroplast from a light green part of the inner leaf of a young shoot.

older green cells (Fig. 7). In some of these dense bodies an inner structure was revealed consisting of what seems to be a net of anastomotic tubules in which a granular osmiophilic substance may be included (Fig. 2).

In young growing cells bodies were also found with a dense core surrounded by a lighter region within an enveloping membrane (Fig. 3). It is in this region outside the dense core that the first laminae are to be observed. A first indication of lamina-formation is revealed as a series of folds of what apparently is a membrane surrounding the dense core of the body. At a later stage of growth the laminae become manifest as extensions of the folds thus giving eventually the double sheet effect of the further developed laminae (Fig. 5). A sectional view of the structure lying outside of the dense core is shown in the upper part of Fig. 4.

Figs. 6, 7 and 8 show subsequent stages of the development of the chloroplast, including a rapid multiplication of the laminae, which appear to be packed into a number of groups (Fig. 7, 9). From this may be inferred that new laminae develop in close association with old ones.

Osmiophilic granules within the chloroplast. From the earliest stages of development there exist clusters of osmiophilic granules in the chloroplast (Figs. 2, 9, 11). These distinctive components have been observed among others by Wolken and Palade in *Euglena* (9).

Occurrence of double laminae. The chloroplast is traversed by thin laminae the thickness of which has been measured from the micrographs as being of the order of 60–80 Å. Significantly there is to be noticed in certain areas a pairing of laminae between which is included a granular substance (Figs. 8, 12). There is a gradual merging of the separate pairs toward the extremities of the chromatophore (Fig. 9) outside of which further appreciable amounts of granular substance is often found. In other central parts of the plastid there can be observed concentrations of this granular substance which also constitutes a thick layer near the external surface of the plastid (Figs. 8, 13). These observations are most clearly delineated in stages not too highly developed. In cells of leaves which have been subject to intense illuminations the chloroplasts often contain accumulations of compact homogeneous material earlier identified with starch grains (Fig. 14).

The chloroplast "grana". In accordance with earlier investigations the "grana" of the chloroplast are characterized by stacked formations of lamellae. These grana are revealed as sharply defined areas of varying form and size within the laminar system. A sectional view through a granum in the lamina-plane is always defined as circular. The number of lamellae in a granum may be quite small or extend to the region of a hundred. In leaves

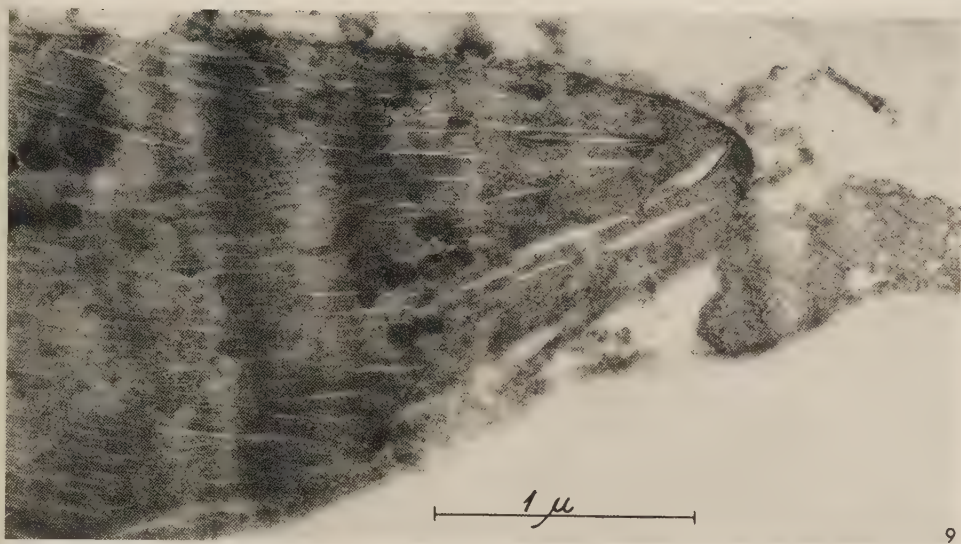


Fig. 9. Chloroplast with groups of laminae.

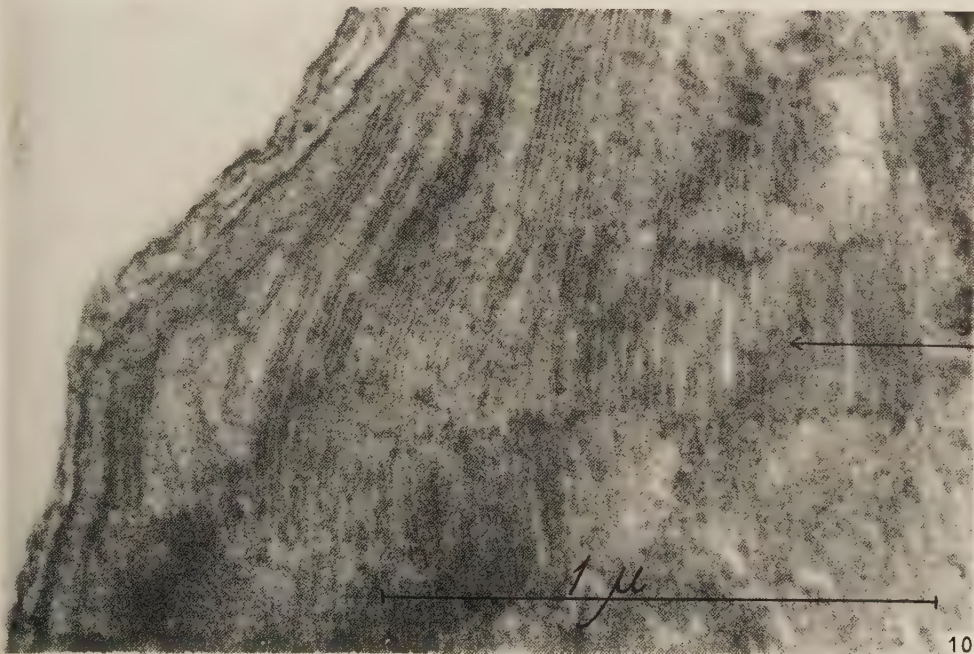


Fig. 10. Granum (g) in a chloroplast of *Aspidistra elatior*.

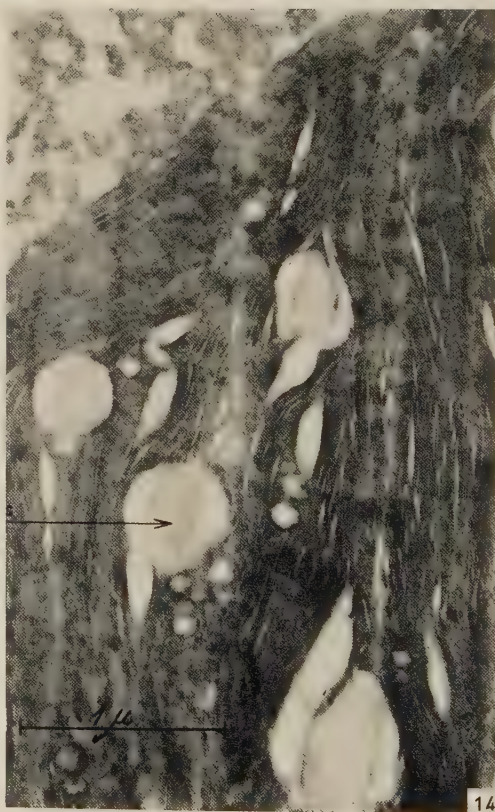
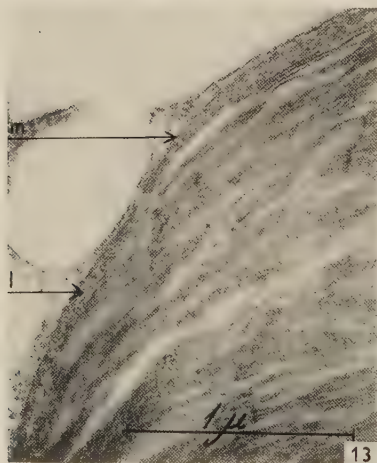
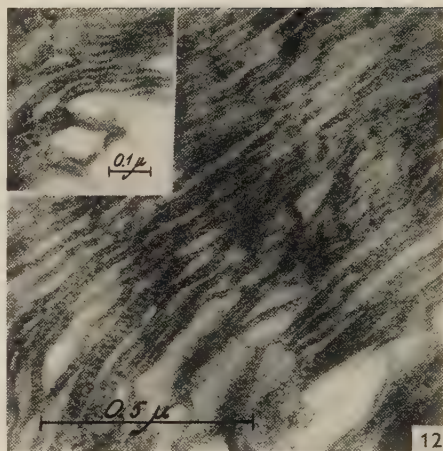
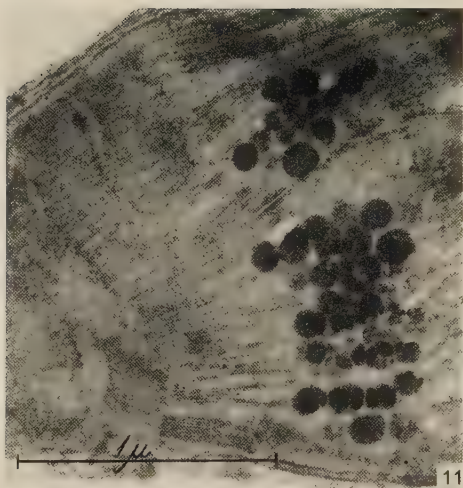


Fig. 11. Osmiophilic granules in clusters within the plastid.

Fig. 12. Paired laminae including granular substance.

Fig. 13. The chloroplast membrane (m) and the granular layer (l) near the chloroplast surface.

Fig. 14. Chloroplast with starch accumulations (s).

which had been exposed to intense light before fixation the grana were more emphasized. Within the granum the lamination is accentuated because of a high degree of osmium impregnation, an even thickness, and a more strictly parallel configuration. At least in some stages, the laminae are not disposed toward twin formation and are devoid of interspersed granular substance. Almost all these laminae extend uninterrupted into the surrounding extragranular regions. The boundary of the closely packed laminae is particularly distinguished by small local concentrations of osmiophilic substance and is further specified by frequent interlaminar anastomoses. The numerical density remains practically unchanged within and without the boundary, but small differences referring to the number of lamellae have been noticed which may be relevant to the further elucidation of grana-structure.

The chloroplast membrane. As can be seen from Figs. 1–5 the micrographs of the chloroplast, especially those for the early stages of development, show an outer layer from which it is possible to construe the existence of a membrane. In somewhat older plastides the outer layer consists of the granulated substance which was described previously. Outside this there can often be seen one or more, usually two, very fine lines indicating a tenuous membrane (Note Fig. 13).

DISCUSSION

Early stages in chloroplast development. The origin of chloroplasts has been studied previously by Strugger with light microscopy (6, 7, 8) and by Leyon with electron microscopy (4). In the latter paper by Strugger an elaborate description is given of the primordial stages of the development of the chromatophores of *Agapanthus*, *Elodea*, and *Chlorophytum*. According to Strugger the proplastids consist of a colourless stroma enveloping a dense primary granum which stains readily with basic dyes, contains chlorophyll and is capable of reproduction.

Strugger's description of proplastids may be traced in Figs. 2–3 of this report, Strugger's primary granum obviously corresponding to the core of the small dense body. Corresponding stages of development have been noted in *Taraxacum* by Leyon (4), in which report micrographs showing division of proplastids may be seen. These Strugger's observations are thus consistent with the results due to electron microscopy. However, so far the exact origin of the small dense bodies, i.e. the primary grana, is quite unknown and therefore the inference by Strugger that Schimper's theory of continuity is thereby established, seems premature.

The concentration of the chlorophyll in the "primary granum" as noted by Strugger is of interest. In this connection attention should be given to the possibility that the osmiophilic granules earlier described in this report do contain chlorophyll.

The chloroplast membrane. In a previous report (3) it has been stated that the evidence given for the existence of a chloroplast membrane was insufficient. (See also Frey-Wyssling and Steinmann (2)). The micrographs now provide further information: the outer granular layer which, in some stages of the chloroplast development (Fig. 8, 13), is especially prominent seems to be restrained by a thin membrane indicated by one or more, usually two, very thin contours, about 50 Å thick as may be estimated from the micrographs.

The chloroplast laminae and their multiplication. As mentioned previously the presence of groups of stacked laminae indicate that new laminae arise in connection with old laminae. The mechanism for this is uncertain so far.

There might be a migration of the granular material within the double sheets (Figs. 8, 12) toward or away from the interior of the plastid. From this it may be inferred that the chloroplast laminae may be regarded as surface-asymmetrical with one side specifically related to the granular substance. This substance may be produced as the result of assimilation.

The hypothetical surface-asymmetry of the laminae as well as the presumed existence of selective paths of transport within the plastid would be compatible with a scheme of lamina-formation logically developed from the facts observed in previous very young stages, i.e. a formation of new connected double sheets through a process of successive protrusions and extensions from local parts of old laminae. However, such a scheme must be considered as hypothetical at the present time, other possibilities being also plausible.

The formation of the stacked laminar grana is an interesting phenomenon but so far data in this respect are insufficient to warrant discussion.

SUMMARY

The development of the chloroplast of *Aspidistra* is similar to that of the *Taraxacum* chromatophore which has been described previously. The first stage observed is a small dense body situated in the protoplast near the cell wall. During growth it differentiates into two parts, one being the inner dense core and the other the surrounding layer of lighter material. The first sign of laminae is manifested as small outward folds of what appears to be some form of an envelope around the core. The grouping of discrete stacked

lamina-formations seem to indicate that new laminae arise in connection with old ones.

The "grana" consist of regularly arranged single lamellae which extend into the extra-granular regions where they become paired each double-sheet including some granular substance. At the point of emergence anastomoses often occur. The development and function of this laminar system is briefly discussed.

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BRIEF NOTES

A ROTARY DISPLACEMENT SPECIMEN HOLDER FOR THIN-SECTIONING WITH RECIPROCATING MICROTOMES¹

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WITH the development of methacrylate polymers as the embedding medium of choice in the preparation of thin-sections for electron microscopy it became important to avoid contact between the specimen block and the knife edge except during the cutting stroke. Selby (8) points out that due to the slight expansion of methacrylate after cutting there is an undesirable wetting of the block and dulling of the knife as a result of contact during the return stroke. In addition, there is a tendency for the specimen block to pick up the previously cut section from the knife edge making it virtually impossible to obtain ribbons. If individual sections are pulled away from the knife edge as they are cut it is difficult to maintain a uniform rate of cutting and sections of varying thickness are likely to result.

Compromise modifications of existing standard commercial microtomes for thin-sectioning have generally failed to eliminate non-cutting knife contact because of their reciprocating action. The recent custom designs, however, recognize this problem and employ such devices as rotating specimen holders (1, 6, 9), inclined planes (4), or back-feeding (5) so that knife contact is accomplished only during the cutting stroke. One new commercial reciprocating thin-sectioning microtome, the Servall, displaces the specimen lateral to a thin glass knife during the non-cutting up-stroke. The Sjöstrand ultra-microtome, which is also available commercially (LKB-Produkter, Stockholm, Sweden), is a rotating specimen holder type.

Recently Dempsey and Lansing (2) described new knife holders, to be used with modified rotary microtomes, which rock back from the cutting plane during the up-stroke of the microtome. These holders, however, move the knife parallel to the axis of the specimen feed and, therefore, any play in the rotating bearings would have a maximum effect on the repositioning accuracy of the knife with relation to the original cutting-plane.

The specimen holder described and illustrated in this paper (Figs. 1 to 4) was designed to provide lateral displacement of the specimen during the up-stroke (Fig. 2) of the microtome when used in conjunction with a glass knife (7). Since the required displacement is at a right angle to the axis of the specimen feed, reposition-

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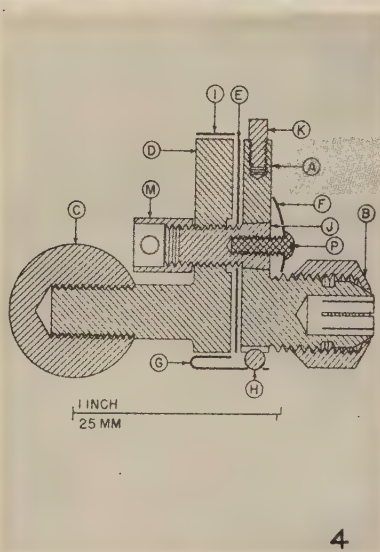
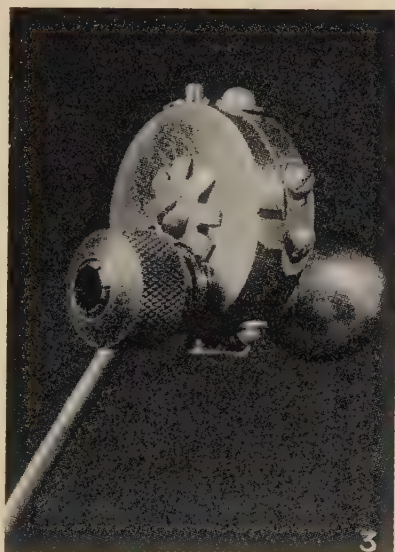


Fig. 1. Position of the specimen holder during the cutting stroke.

Fig. 2. Position of the specimen holder during the return stroke.

Fig. 3. Assembled rotary displacement specimen holder for International Minot rotary ultra-thin sectioning microtome.

Fig. 4. Cross section assembly drawing for rotary displacement specimen holder. Legend: A - chuck-plate; B - chuck-tightener; C - support-ball; D - support-plate; E - bearing-washer; F - star-washer; G - locking-ball spring; H - locking-ball; I - stop; J - taper-pin; K - stop-pin; M - capstan nut.

ing errors relative to the cutting plane are kept at a minimum. Accuracy of cutting-plane alignment is maintained, as diagrammed in Fig. 4, by spring loaded (F) seating of the surface-ground inner surfaces of the chuck-plate (A) and support-plate (D). Rotational accuracy is achieved by the adjustable taper-pin (Fig. 4, J).

The specimen holder illustrated was constructed by one of the authors (S. W.) who has limited machine shop experience. The design simplicity and ease of construction are evident. All the necessary materials are standard and easily obtainable making construction a relatively easy job for any qualified machinist.

The rotary displacement specimen holder was designed for use with an International Minot rotary ultra-thin sectioning microtome (3). In adapting it to other microtomes the length, shape, or position of the actuating rod may have to be modified to provide suitable working clearance. Likewise, the support-ball (Fig. 4, C) could be replaced by any other configuration needed for secure mounting to the specimen slide-block of the microtome in use.

An automatic actuating mechanism was originally contemplated which would have linked the actuating rod of the specimen holder to a moving part of the microtome. This would have provided mechanical synchronization of lateral displacement with the cutting cycle of the microtome. However, experience with the specimen holder has shown that sections and ribbons of satisfactory uniformity, down to $0.05\ \mu$, are easily obtainable with hand operation. With a little practice the cutting cycle can be repeated at a very uniform rate. For those who desire an automatic actuator it should not be difficult to devise a simple linkage for most microtomes.

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REACTIONS OF UNFERTILIZED MOUSE EGGS TO SOME EXPERIMENTAL STIMULI

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INFORMATION is available in the literature on the reactions of unfertilized rabbit (8, 9) and rat (1, 2, 9) eggs to experimental stimuli applied *in vivo*, but is lacking for mouse eggs. The present communication describes the effects on mouse eggs of the application of heat and cold *in vivo*, and of ether anaesthesia.

Hot-shock treatment was applied to the eggs by bringing the ovaries and fallopian tubes to the exterior through dorsolateral incisions in the body wall, and immersing them in a water bath maintained at 44.0–45.0° C. Nembutal was administered subcutaneously as an anaesthetic, and the treatment was applied for 5–10 min. about 8–12 hr. after ovulation.

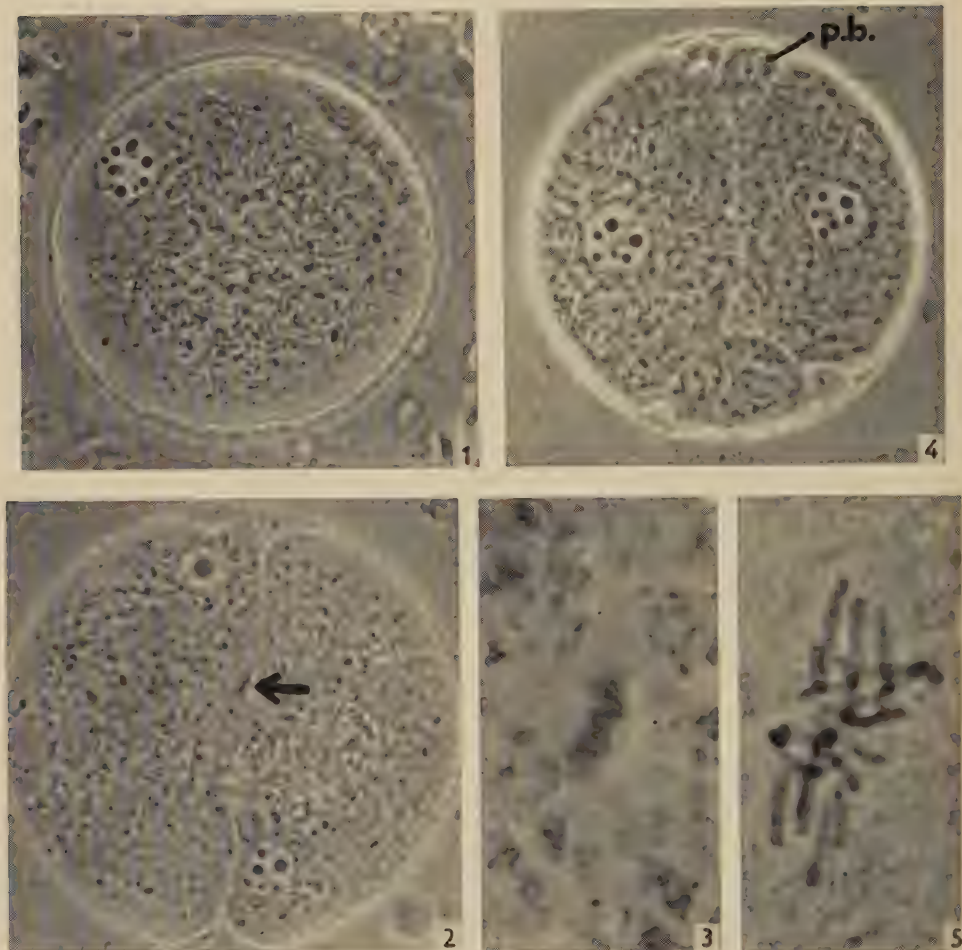
Ten mice killed 4–5 hr. after hot-shock treatment yielded a total of 67 eggs. Eight eggs exhibited no change; the reactions shown by the remainder were of three different types, as follows:

1) 26 eggs (39 per cent) showed degenerative changes and need not be further considered.

2) 28 eggs (42 per cent) showed reformation of nuclei, all of which resembled the pronuclei of fertilized eggs (Fig. 1). A single nucleus was present in all the eggs except for one in which two nuclei could be seen. In 21 mononucleate eggs polar bodies were identified and it was considered that abstriction of the second polar body had occurred in most instances; these eggs would therefore be haploid. Abstriction of the second polar body and re-formation of nuclei have also been reported in rat eggs, but only after cold shock treatment (2). The egg with two nuclei had no polar body, and it is likely that these features developed in a manner similar to that described in heat-treated, fertilized *Triturus* eggs (6), namely, through the completion of the meiosis without polar body abstriction. The occurrence of two female pronuclei and no second polar body has also been noted in mouse and rat eggs that were undergoing fertilization (1, 3, 5).

3) Five eggs (7.5 per cent) had undergone a form of cleavage, for they were each composed of two cells which contained apparently normal nuclei (Fig. 2). In two eggs cytoplasmic division had been unequal, but in the other three eggs, the two cells were of similar size and the eggs could not readily be distinguished from fertilized 2-cell eggs. The occurrence of these cleft eggs so soon after the application of the stimulus is remarkable and an explanation of it should clearly be attempted. It

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All photographs taken with phase-contrast microscope. In Fig. 1–4 the eggs are seen in the fresh state, and moderately compressed between cover slip and slide. Fig. 5 is of a squash preparation after fixing and staining.

Fig. 1. Single well-formed nucleus in mouse egg recovered after hot-shock treatment ($\times 400$).

Fig. 2. A 2-cell egg formed by the process referred to as "immediate cleavage". The residue of a spindle can be seen, indicated by the arrow ($\times 400$).

Fig. 3. Enlargement of part of Fig. 2, to show spindle remnant more clearly ($\times 1600$).

Fig. 4. Induced 2-cell egg recovered 48 hr. after hot shock treatment, showing apparently normal nuclei, a polar body (p.b.), and two or three cytoplasmic extrusions ($\times 400$).

Fig. 5. Haploid prophase chromosome group in 2-cell egg recovered after hot shock treatment ($\times 1350$).

seems significant that none of these eggs had a recognisable second polar body, and that an object that was almost certainly the central body of the second meiotic spindle could be identified between the two cells in each of the eggs (Fig. 2 and 3). The evidence therefore suggests that, following hot-shock, the spindle migrated towards the centre of the egg and completion of the meiosis in that position induced a cytoplasmic cleavage that virtually bisected the egg. The two chromosome groups then formed nuclei and thus completed the resemblance to 2-cell fertilized eggs. The fact that the cells were very different in size in two of the eggs is also consistent with the idea that these 2-cell eggs, seen so early after hot-shock, were formed by an aberration of the process normally involved in polar-body abstriction. One of the two cells, even in the equally divided eggs, therefore corresponds to a polar body, and the nuclei in these cells can be deemed to represent female pronuclei. For convenience, the process whereby these 2-cell eggs are thought to be formed, is referred to as "immediate cleavage".

Ten mice were killed 48–70 hr. after the hot-shock treatment, and a total of 74 eggs was recovered. Gross fragmentation was shown by 55 eggs; the remaining 19 eggs resembled in nuclear form and in the evenness of the blastomeres the 2-cell stage (15 eggs) or the 4-cell stage (4 eggs) of fertilized eggs, except that in some of them small cytoplasmic bodies were also present (Fig. 4). Polar bodies could be identified in some of the 2-cell eggs, and these, therefore, were probably not formed by the process of "immediate cleavage", but resulted from the division of mononucleate eggs. The resemblance of the 2-cell and 4-cell eggs to cleavage stages of fertilized eggs suggests that they could have developed even further and it is perhaps significant that Fischberg and Beatty (8) have reported the identification of a haploid mouse egg in the 33-cell stage after hot-shock treatment in mated mice.

The effect of ether anaesthesia on mouse eggs was tested by deeply anaesthetizing 12 mice for 15 min. about 10–14 hr. after ovulation. The animals were killed either 4–5 hr. or 19–20 hr. after treatment. A total of 98 eggs was recovered, of which 7 eggs exhibited a single well-formed nucleus and a second polar body, 9 eggs were in the 2-cell stage and one egg was in the 4-cell stage. This 4-cell egg and 8 of the 2-cell eggs were obtained from two mice killed 19 hr. after treatment. It is considered that all, or nearly all, of them were examples of the phenomenon just described as "immediate cleavage". In two of the 2-cell eggs, the nuclei had given place to prophase or metaphase chromosomes (Fig. 5). When these two eggs were fixed, stained and squashed, it was found possible to count the number of chromosomes in 3 of the 4 cells; the results were 20 ± 1 , 18 ± 2 , 18 ± 2 , indicating that the eggs were haploid ($2n = 40$).

Cold-shock treatment has been found very effective for inducing extrusion of the second polar body in rat eggs (1, 9) and a small proportion of such eggs have also been found to exhibit nucleus formation and cleavage (2). The technique was therefore applied to 10 mice. The animals were anaesthetised with nembutal about 8–12 hr. after ovulation and ice was applied to the tubes for 1 min. A total of 64 eggs was recovered 4–18 hr. later. There was no evidence of activation: in 27 eggs the metaphase of the second meiotic division was still visible, 11 eggs were fragmented, and in the remainder neither chromosomes nor nuclei were seen.

The mouse egg thus differs from rat and rabbit eggs in its reactions to heat and cold. In response to cold shock the rat egg regularly extrudes the second polar body

and both rat and rabbit eggs may show nucleus formation; the mouse egg showed neither reaction. Hot-shock treatment inhibits the second meiotic division in the rat egg (1), but evokes it in the mouse.

Briefly, the results of the investigations just described are as follows: Hot-shock treatment and ether anaesthesia are capable of inducing nucleus formation in unfertilized mouse eggs. Second polar bodies are formed in most instances, so that the eggs are almost certainly haploid. Seemingly normal 2- and 4-cell stages have also been seen and further development may be possible. A surprising reaction, referred to as "immediate cleavage", was the formation, within a few hours of treatment, of 2-cell eggs that closely resembled in cytoplasmic and nuclear form the 2-cell fertilized egg. These eggs were shown to be haploid; their formation probably involved completion of the second meiosis near the centre of the egg. One of the cells would thus be genetically different from the other, or, in other words, the egg would be a genetic mosaic. The nuclear and nucleolar volumes of some of the nucleated 1-cell and 2-cell eggs described in this communication have been determined; the results and their significance in relation to conditions influencing nuclear size and form are discussed elsewhere (4). Mouse eggs resemble rat eggs in that they may be activated by ether anaesthesia, but differ from both rat and rabbit eggs in showing little response to cold-shock treatment.

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A SIMPLE MICROCUVETTE ADAPTED TO THE BECKMAN SPECTROPHOTOMETERS

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THE modified absorption cell devised by Lowry and Bessey (4) for use in the standard cell holder of the Beckman spectrophotometers (Beckman Instruments, Inc., South Pasadena, California) has a path length of 10 mm and requires about 25-50 μ l of solution. Although the cell is sensitive, accurate, and convenient to use, the necessary volume has frequently been unattainable in making certain measurements on enzyme activities of small parts of the early chick blastoderm. For microspectrophotometry, Holter and Løvtrup (3) and Krugelis (2) have employed, on the microscope photometer described by Commoner (1), cylindrical cells of heavy-walled capillary tubing (with a microscope slide as entrance window and a cover slip as exit) which have path lengths of 5-7 mm and capacities of only 5.5 μ l. Somewhat larger varieties of this design have been used in the Beckman model B spectrophotometer, with the aid of the brass adapter described below, and have given results comparable in reproducibility with those obtained with standard cuvettes.

When initially set up, the brass adapter (A in Fig. 1) is placed with a piece of black paper or foil (B) into a position of the Beckman cell holder No. 5010 (C). A diaphragm opening (D) is then pierced into the paper through a segment of capillary tubing, identical with that comprising the microcell to be used, held in place with the set screw (E); the diameter of the opening may be as large as the bore of the cell. The orientation of holder, adapter, and diaphragm is permanent until another cell may be brought into use. The cell itself (F) consists of heavy-walled capillary tubing of 1.0 to 2.0 mm bore, cut off to 5 or 10 mm length on a glass-worker's abrasive wheel or broken off approximately and ground to length by hand on a wet carborundum stone. The front window (G) is a fragment of No. 1 cover slip (a quartz cover slip may be used if desired) cemented to the cell with a light coating of shellac. After the cell is baked for a few hours at 90° C, the shellac seal is virtually unaffected by ordinary aqueous solutions. For a series of readings, the cell is placed in the adapter and held by the screw with the sealed window against the diaphragm opening. This arrangement is not altered until the readings are completed and the cell is removed for rinsing. The cell is filled in the assembly by pipetting in solution to form a slightly convex surface, and the rear window (H, another fragment of cover slip) is dropped on with care to avoid bubbles. The assembly is then placed in a reproducible manner in the Beckman carrier (which may have to be adjusted slightly for maximum transmission) and balanced against the desired blank solution held in a standard cuvette. The optical densities of the solutions in the microcuvettes have ordinarily been read at slit widths of 0.1 to 0.3 mm (band widths of 2-3 $m\mu$).

at sensitivity 3 or 4; lower sensitivity settings may be used with larger slit widths. To replace the solution, the assembly is taken from the carrier and only the back window removed.

In preliminary tests, absorbance readings on single samples did not vary more than 0.01 unit (S.D. 0.005) during repeated repositioning of the carrier. Means among different samples varied less, so that changing the cover slip has essentially no effect

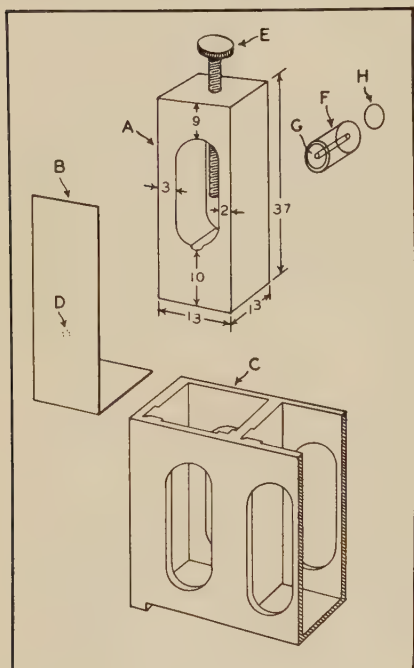


Fig. 1. The disassembled microcuvette, brass adapter, diaphragm paper, and Beckman cell holder. Approximate dimensions are given in millimeters. Explanation in text.

on the optical properties of the assembly. It has been pointed out (4) that carrying the diaphragm opening with the cell assembly, as in the present procedure, produces variations caused by lateral differences in spectral quality and phototube response. Even more important at narrow slit widths is the variation in the vertical length of the light beam intercepted by the upper and lower edges of the diaphragm. These difficulties are precluded, however, if the cell holder is placed in the carrier in a standardized position and the notched carrier guide is always set with care. A great advantage of the present microcell, in addition to the moderate gain in sensitivity due to its decreased volume without sacrifice of path length, is that it is notably inexpensive and may be manufactured in the laboratory. The cells, moreover, are versatile in that quartz windows may be cheaply employed; air may be more or less excluded for certain enzymatic reactions; and, in models which have been made with double side arms and both windows sealed, mixing of solutions and changing of the atmosphere is readily accomplished.

The author thanks Mr. Nelson Krause of the Department of Biology, The Johns Hopkins University, for machining and aid in the design of the brass adapter; and Mr. John S. Spurbeck for the illustration.

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ACTION POTENTIALS IN SEA URCHIN EGGS AT FERTILIZATION¹

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THE similarity between the process of activation in sea urchin eggs and other excitatory processes in plant and animal cells has led many to speculate whether similar mechanisms are involved. One of the most generally observed features of response mechanisms is an action potential; such a potential has not previously been found in sea urchin eggs. Peterfi and Rothschild (6) reported transient electrical changes at the surface of the frog egg on fertilization, but Rothschild (7) was not able to measure a membrane potential in either unfertilized or fertilized eggs of sea urchins. He notes that this failure may be ascribed to electrical leaks around the electrodes, but on the basis of indirect evidence concludes that an action potential is unlikely in this egg (8). The recent success of the microelectrode technique in measuring resting and action potentials in nerve and muscle led us to attempt similar measurements in sea urchin eggs.

The eggs of *Paracentrotus lividus* and *Arbacia lixula* were used in this study. In order to insert microelectrodes into these eggs successfully, it was found necessary to remove the jelly coat by brief treatment with acidified sea water (10). Electrodes were prepared from Pyrex glass tubing with the aid of a simple mechanical device: The tubing was drawn by hand to an outer diameter of about 1 mm. The butt end of the tubing was then fixed on a cork mounted on a ring stand, and a hook was bent in the end of the thin shank. Tension was then applied by means of an ordinary elastic band fastened to the base of the ring stand, and a microflame applied carefully just above the hook. By adjusting the tension of the elastic band electrodes of any desired diameter could be rapidly drawn in this way. The electrodes were then filled with sea water; KCl proved unsatisfactory because cytolysis often followed insertion of KCl-filled electrodes into the eggs. Connection to the recording apparatus was made through Ag-AgCl electrodes soldered to shielded copper cables.

For potentiometric measurements, the eggs were mounted in sea water on a glass slide, and a microelectrode of diameter 5 μ or less inserted into the egg with a micro-manipulator. A second electrode of 1 mm diameter was placed in the sea water

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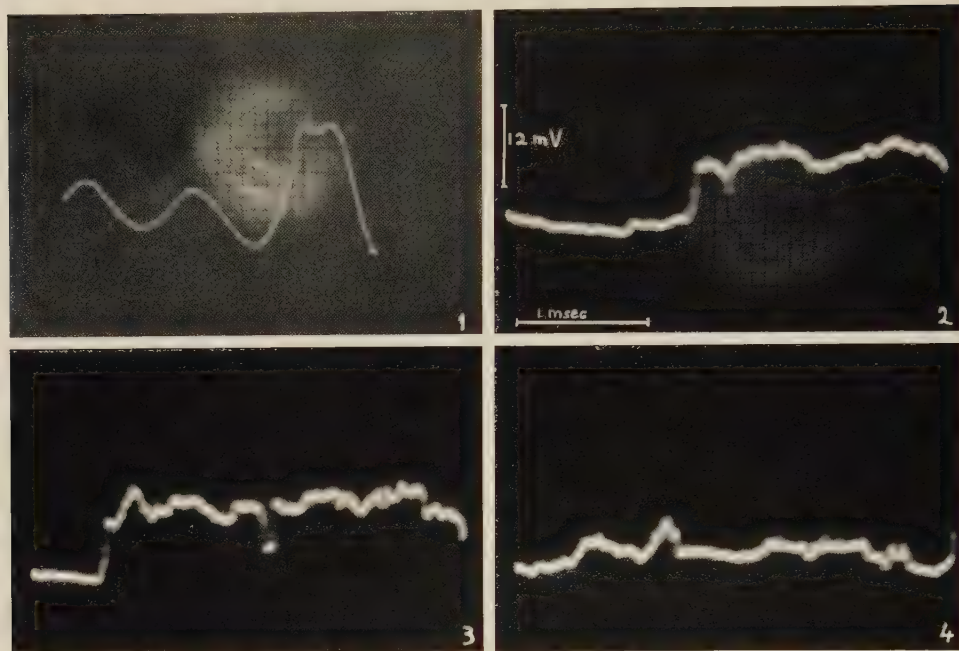
medium. The terminals of the electrodes were then connected to an electrometric vacuum tube potentiometer, range 0–100 mV. For oscillographic measurements, the same arrangement was used, but the electrode in the medium was grounded and the entire system of microscope, electrodes and micromanipulator enclosed in a metal box, with apertures only for the microscope eyepieces and for illumination; the box was grounded to a common ground with the indifferent electrode and the recording equipment. For measurements of action potentials, the microelectrode was inserted into the egg, the microscope adjusted, and after the eggs were inseminated the box was closed. No further manipulations within the box were made during the period of recording. In this way, extraneous impulses were effectively excluded; moreover, the major observations were made at a time when the laboratory was not in use for other work, to obviate interference from external sources.

Potentials were recorded photographically on the screen of an oscilloscope tube operated with driven sweep. The input signal was amplified by means of a symmetrical linear amplifier having a gain of 10^4 . Because of the high resistance (ca 10^7 ohms) and parallel capacitance (ca $1 \mu\text{F}$) of the recording electrode, the first stage of the amplifier was planned to give good fidelity with a high input impedance. The rise time of the amplifier was about $20 \mu\text{sec}$.

We never observed definite membrane potentials in the eggs used. There was no potential change, with either potentiometric or oscillographic recording, when the microelectrode was inserted into or withdrawn from the egg. In some instances, with *Paracentrotus* eggs, a layer of cortical material could be seen surrounding the tip of the electrode; in such instances it appears unlikely that electrical contact with the cytoplasm was made. In other cases with *Paracentrotus*, and invariably with *Arbacia*, no such layer could be seen, and the electrode, when withdrawn, brought a thread of cytoplasmic material with it. The latter cases in general gave the most satisfactory action potential measurements, and we feel confident that the microelectrode was in actual contact with the cytoplasm. It has generally been observed in work with nerve and muscle that microelectrodes give correct measurements of resting potentials even when inserted through the cell membrane. In view of the readiness with which the membrane of the sea urchin egg reforms after injury, we consider that electrical leakage around the microelectrode is unlikely, and therefore agree with Rothschild's conclusion (7) that the unfertilized sea urchin egg does not exhibit a membrane potential measurable with our methods.

When the impaled egg was fertilized, there appeared, in all cases where good electrical contact appeared to be established, and a vigorous fertilization reaction could be observed, a definite potential change. In the electrometric studies the potential difference appeared suddenly, approximately coincident with the first visible changes in the egg (cortical reaction). The difference amounted to between 2 and 5 mV, with the cytoplasm positive to the medium. It persisted during the period of egg contraction, and then gradually returned to zero.

With oscillographic recording, we observed a series of rapid pulses, beginning simultaneously with or shortly after the first visible changes in the egg surface. The form and number of these pulses were somewhat different in the two species studied. In *Paracentrotus* eggs, the pulses were usually few in number; often only one or two were observed, even in eggs where good electrical contact appeared to have been made. They were also quite regular in form, consisting of a simple damped



Oscillographic recordings of the action potentials at fertilization.

Fig. 1. *Paracentrotus lividus* (slightly retouched).

Figs. 2-4. *Arbacia lixula* (unretouched): three successive recordings. The scale on Fig. 2 applies only to *Arbacia*.

oscillation of rise time about 0.1 msec and magnitude about 5 mV or more (Fig. 1). In *Arbacia* eggs the pulses were usually quite numerous, as many as fifty having been observed in one sequence. They were also quite irregular in form, with a marked oscillatory character (Figs. 2-4). In both cases, however, the pulses did not continue after contraction of the egg was completed. It should be noted that this difference in electrical properties of the eggs of the two species is paralleled by differences in the changes in optical properties which follow fertilization (3).

It is not possible from our results to arrive at definite conclusions concerning the origin of the observed potentials. It is clear, however, that the action potential of the sea urchin egg is not strictly comparable with that of nerve or muscle. The absence of a resting potential, and the fact that sea urchin eggs exhibit no measurable change in resistance during fertilization (1), taken with the oscillatory character of the egg action potential demonstrate this clearly. Evidently the egg potential involves the development, following fertilization, of transient electromotive forces. These electromotive forces may be associated with, and the potential resulting from their action is very probably modified by, the changes in the ultrastructure of the surface evidenced by the increase in capacitance (2) and the change in optical properties of the cortex (3, 4, 5, 9).

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THE STRUCTURE OF CHLOROPLASTS

V. *CHLORELLA PYRENOIDOSA* PRINGSHEIM STUDIED BY MEANS OF ELECTRON MICROSCOPY

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IN connection with other studies in the general field of chloroplast structure and related cytochemical properties the alga *Chlorella pyrenoidosa* Pringsheim has received some attention with regard to its constitution as revealed by light and electron microscopy.

The strain investigated was obtained from The Institute of Food Chemistry, the Royal Institute of Technology, Stockholm, and was labelled F. E. Allison, U.S.D.A., strain L 72 a. It was cultured in a conventional medium using continuous illumination and vigorous stirring; a mixture of air and a few per cent CO₂ bubbling through the solution.

The accompanying micrographs are of a *Chlorella* suspension which had been growing intensely for 6 days.

Concentrated sucrose solution was added to a sample of the algae-suspension to give a final molar concentration of 0.2. The fixing material of 2 per cent OsO₄ dissolved in water was held in a small glass cup, the top of which was closed by a microscope slide with a small drop of the sucrose-sustained sample hanging from underneath in the fixative vapor. The fixation proceeded for 2-4 hours at room temperature in darkness. The technique of dehydration, embedding and sectioning has been described in a previous report (3). The isolation and collection of the algae when changing the medium was accomplished by centrifugations.

Chlorella vulgaris Beyer has been previously described by both Artari (1) and Grintzesco (2) as rotund or oval cells with a central nucleus and a cupshaped chromatophore in which a pyrenoid can usually be discerned.

The present observations reveal a thin cellular wall surrounding the cupshaped chromatophore containing the pyrenoid. Situated within the chromatophore is the nucleus with its nucleol. In the protoplast rounded grana of varying size and very intensely blackened by the osmium fixative were usually found.

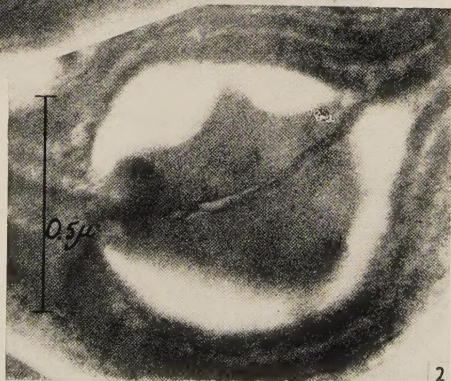
The wall of the cupshaped chromatophore consists of a number (4-8) of curved connected laminae with a thickness of about 35 mμ.

Morphologically the pyrenoid appears to be quite simple seemingly formed by a local thickening of two adjacent laminae. The extreme parts of the pyrenoid may also be in contact with the immediate laminae on each side. Except for the line of partial and symmetrical cleavage no fine structure could be distinguished in the pyrenoid.



Fig. 1. Section through *Chlorella pyrenoidosa*, showing the cell wall, the cupshaped chromatophore with its pyrenoid (py) and the cell nucleus (n), 57000 $\times$.

Fig. 2. The pyrenoid, 75000 $\times$.



On the other hand the laminae of the chromatophore clearly showed a fine structure and itself appeared to consist of some, usually four, very thin layers of a thickness of the order of 50 Å. Thus this chromatophore bears a striking resemblance to that of *Fucus* (4).

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